CONNECTIVE TISSUE

A SYMPOSIUM

organized by

THE COUNCIL FOR INTERNATIONAL ORGANIZATIONS OF MEDICAL SCIENCES

Established under the joint auspices of UNESCO *and* WHO

Edited under the direction of

R E TUNBRIDGE

The University of Leeds

by

MADELINE KEECH
The University of Leeds

J. F DELAFRESNAYE
C.I.O.M.S., Paris

G. C. WOOD
The University of Leeds

BLACKWELL
SCIENTIFIC PUBLICATIONS
OXFORD

© *Blackwell Scientific Publications Ltd*, 1957

This book is copyright. It may not be reproduced by any means in whole or in part without pe
Application with regard to copyright should be addressed to the publishers.

Published simultaneously in the United States of America by Charles C Thomas, Publisher, 3
East Lawrence Avenue, Springfield, Illinois

Published simultaneously in Canada by The Ryerson Press, Queen Street West, Toronto 2

FIRST PRINTED FEBRUARY 1957

PRINTED IN GREAT BRITAIN IN THE CITY OF OXFORD
AT THE ALDEN PRESS
AND BOUND BY THE KEMP HALL BINDERY, OXFORD

CONTENTS

LIST OF PARTICIPANTS

G. Asboe-Hansen	University of Copenhagen (Denmark)
W. T. Astbury	University of Leeds (U.K.)
J. Baló	University of Budapest (Hungary)
I. Banga	University of Budapest (Hungary)
Suzanne Bazin	Institut Pasteur, Garches (France)
R. S. Bear	Massachusetts Institute of Technology (U.S.A.)
Joane H. Bowes	The British Leather Manufacturers' Research Association (U.K.)
R. Consden	Canadian Red Cross Memorial Hospital (U.K.)
A. Delaunay	Institut Pasteur, Garches (France)
T. Gillman	University of Natal (Union of South Africa)
L. E Glynn	Canadian Red Cross Memorial Hospital (U.K.)
W. Grassmann	Max-Planck-Institute for Protein and Leather Research, Munich (Germany)
J. Gross	Harvard University (U.S.A.)
K. H. Gustavson	Swedish Tanners' Research Institute, Stockholm (Sweden)
D. A. Hall	University of Leeds (U.K.)
Sylvia Fitton Jackson	King's College, London (U.K.)
D S Jackson	University of Manchester (U.K.)
K Meyer	Columbia University College of Physicians and Surgeons (U.S.A.)
Helen Muir	British Postgraduate Medical School, London (U.K.)
A. Neuberger	St. Mary's Hospital Medical School, London (U.K.)
V N. Orekhovitch	Academy of Medical Sciences of the U S.S R., Moscow (U S S.R.)
S. M. Partridge	Low Temperature Research Station Cambridge (U K.)
R. Reed	University of Leeds (U.K.)

A. H. T. Robb-Smith	Oxford University (U.K.)
W. Schwarz	The Free University of Berlin (Germany)
O. Snellman	Uppsala University (Sweden)
B. Sylvén	Karolinska Sjukhuset, Stockholm (Sweden)
R. E. Tunbridge	University of Leeds (U.K.)
A. van den Hooff	University of Amsterdam (The Netherlands)
F. Verzár	University of Basle (Switzerland)

FOREWORD

The symposium on 'Connective Tissue' was held in London from July 22nd to 26th, 1956, under the chairmanship of Professor R. E. Tunbridge. The meeting followed the general pattern of previous multi-disciplinary symposia run by the Council. For the first time, however, scientists from Soviet Russia and Hungary accepted our invitation.

It is perhaps appropriate to mention that the C.I.O.M.S. groups some fifty international organizations devoted to the sciences basic to medicine and to the clinical branches. Its aim is to improve communication between disciplines and across national boundaries. Because of its constitution, the Council receives many suggestions for symposia, these come from the international organizations composing its membership, from national research councils or national medical societies and from individuals interested in the Council's work.

Among many topics the various aspects of the biology of connective tissue were suggested for the Council's 1956 symposium. After careful consideration, it was agreed that the meeting would centre on the biochemistry of connective tissue and on the correlations between biochemistry and structure.

The selection of participants was left to the chairman in consultation with the Council's Executive Committee. Every effort was made to make the meeting as representative as possible, both scientifically and geographically. From among the many research workers who, in various countries, are studying connective tissue, a difficult and somewhat arbitrary choice had to be made.

Official languages were English and French, but it soon appeared that English allowed communication between all participants. It is fitting to thank all those who willingly agreed to express themselves in a language which was not their own in order to help the meeting along. In this book, all papers are published in English with the exception of the one by Dr. Delaunay and Mlle Bazin but a summary in English follows the paper.

All discussions were immediately transcribed from magnetic recordings by Dr. Geoffrey Wood and Dr Madeline Keech to whom thanks are due. The discussions are printed in précis fo

at the end of each paper. Papers and discussions are to be taken together just as they were delivered and discussed. Much of the cut and thrust of debate has necessarily been lost for which we are sorry – but the irrelevant and the repetitious has been eliminated in order to present the reader with the substance of the discussion.

We hope that this monograph will be of use to all who study the biology of connective tissue.

INTRODUCTION

W. T. Astbury

> In nature's infinite book of secrecy
> A little I can read
> *Antony and Cleopatra*, Act I, Scene 2.

When our Chairman kindly invited me to contribute a general introduction to this symposium and presented me at short notice with a formidable collection of summaries of papers to be contributed by the other participants, my first reaction, I confess, was to recall Sydney Smith's famous remark 'I never read a book before reviewing it, I find it prejudices the mind.' Here now, I thought, was a beautifully analogous situation where to follow Smith's example was most strongly indicated. I have compromised though – and in the way in which I suppose I was expected to, that is to say, I have of course read the summaries, but this brief assessment of how we stand at the moment with regard to the connective tissue problem is for the most part only as I see it personally. Which means also that the bad parts are mostly mine too.

We would all agree, I believe, on the [illegible]

connective tissue system as a kind of unit – a combination that is to a first approximation self-contained and indivisible. We imagine the cells manufacturing a fundamental matrix, which then acts as site and precursor for the fibre complex that is the principal, and relatively stable, end-product of the system. And always, *ex hypothesi*, we search for common factors, common manifestations. The chief motive in fact of a meeting such as this must be, directly or indirectly, consciously or unconsciously, to inquire again what are these common features and to examine once more how far we have gone in their characterization.

Since it is still very much biogenesis and the primary synthetic steps which are the most difficult and mysterious, the procedure of least resistance in molecular biology generally, and in the connective tissue problem in particular, is to work mostly backwards from the

more permanent end-products – in this case the fibre complex of collagen, elastin, reticulin and anything else there may be, on which indeed the idea of connective tissue unity is just now most clearly focused. This feeling for a common plan underlying the fibrous end-products of the connective tissue system derives from the molecular level of X-ray diffraction analysis, which first brought about in pre-war days an astonishing simplification among the apparently enormous diversity of fibrous proteins. It was found that, in terms of polypeptide-chain types, there are in the main only two configurational schemes, one comprising the long-range elastic fibres of the keratin-myosin-epidermin-fibrinogen group (k-m-e-f group), and the other the inelastic fibres of the collagen group. The collagen group was named after its most familiar member, orthodox collagen of classical histology, but perhaps a little deceptively, because it transpired that one of the most impressive things about the group was its very wide range, including as it does numerous structures previously considered to have little or nothing to do with one another – for example, white connective tissue fibres, tendon and cartilage, the scales and fins (elastoidin) of fishes, the ichthyocol of swim-bladders, the cuticles of Annelid worms, the filaments ejected by the sea-cucumber, jelly-fish, to quote only a selection. They all [illegible] kind of large-angle X-ray

[illegible] peptide-chain configuration they all have in common, still remains the only sure criterion yet discovered of their fundamental family relationship. Many of them give also the collagen small-angle X-ray diagram, corresponding in period (about 640 Å) to the bands seen in the electron microscope, but this is not an essential but is associated with the next stage of organization: the small-angle X-ray diagram

of the earthworm's cuticle), but the large-angle X-ray diagram persists.

Here, beyond doubt, is one of the major designs of molecular phylogeny, comprehensive enough to take in the connective tissue fibres in its stride, so to speak – that is, potentially; for whether it [illegible] demonstrated. I myself

greater unity; that, in other words, it should constitute a specialized subgroup of the collagen group something like the feather-keratin subgroup of the k-m-e-f group. After all, now that the reticulin fibres have been found to satisfy basic collagen-group requirements, only elastin remains to be accounted for, and even that too, these days, is at last beginning to show signs of conforming.

I think, therefore, (the odds in support of the suggestion being at least two to one), that I might very usefully devote much of the rest of this introduction to summarizing some of the findings and conclusions lately arrived at regarding that innermost question of the collagen group from which all else probably spreads out automatically. I refer to the constitution and curious polypeptide-chain configuration which gives rise to the characteristic X-ray diagram and which has offered such long resistance to attempts at elucidating it. This really is the central problem; for let me emphasize that the master plans of the fibrous proteins revealed by X-ray analysis are type-specifications, or themes, susceptible of many variations; and [illegible]

and concepts of that kind – with comparative ease, or at any rate much more confidently.

The new outlook in recent years on possible polypeptide-chain configurations is inspired, as everyone knows, by what may be called the helical interpretation of Pauling and Corey, according to [illegible]

is only the bare bones of the k-m-e-f story, the complete explanation is an elaboration that has not yet been fully worked out.) In the β-form of the k-m-e-f group the polypeptide chains are pulled out almost straight, so that the average length of an amino-acid residue [illegible]

further detail, but I want to make the point that, of the two principal configurations I have just mentioned, the 3.6 α-helix (it is one of a

gether by hydrogen bonds linking CO and NH groups in adjac
chains – they are now all *inter-chain* bonds. What other possibili
are there that might be identified with the collagen configurati
which, as I have said, is so curiously distinct from either of these tv
After the triumph of the α- and β-configurations it would seem r
too difficult to advance thence on collagen and, with the aid of t
elegant diffraction treatment given by Cochran, Crick and Va
(1952), to clear it up similarly, but as a matter of fact it has prov
surprisingly obstinate. Ever since the early days when the collag
group was first recognized, we at Leeds have argued that the collag
chains are practically inextensible for special stereochemical reasc
connected with the preponderance of proline and hydroxyprol
residues; that actually they are *shorter* than in the β-configuration
the extent that the length per residue in the direction of the fib
axis has now fallen to as low as 2.86 Å; but what exactly was t
nature of this constriction? That was always the question; ar
even with the advent of the new helical ideas, as applied b
Pauling and Corey themselves, by Bear, and by Randall and co
workers, for instance, it still turned out far from easy to decide wh
particular form of helix or combination of helices best agreed wi
all the X-ray data (recently made more precise by improved di
grams at the hands of the Randall school) and supporting observa
tions.

The break came eventually through papers by Ramachandra
and Kartha (1954, 1955, 1956), Cowan and McGavin (1955
Cowan, McGavin and North (1955), Crick and Rich (1955), an
Rich and Crick (1955). What it amounts to is roughly this, tha
Ramachandran and Kartha first proposed a structure along th
desired lines, and it has seemingly been improved and rationalized
so to say, by the other authors in the light of X-ray analyses o
poly-L-proline (Cowan and McGavin) and of the hitherto obscur
crystallographic modification of polyglycine (Crick and Rich) tha
has been named polyglycine II (in polyglycine I the polypeptide
chains are i ... It was found that in
poly-L-prol
the same –
pying a length of about 3.1 Å (though the six-fold system o

and the well-known piece of chemical analytical information that one-third of collagen consists of glycine residues and at least another quarter of proline and hydroxyproline residues, then led irresistibly to the conclusion that the collagen structure is simply the ... is ... It ... ical distortion thereof, a so-called coiled coil.

One takes a triad of helices from the polyglycine-polyproline structure (there are two ways of doing this) and twists the three chains slowly round one another so that the number of residues per turn, with respect to the common axis, becomes 3⅓. The period is 2 86 Å and corresponds to three turns. So at last our original ...

The answer to the question I put a moment ago as to what other ...

is, before adaptation) a helix held in that shape by inter-chain bonds, wh... clas... resi... stru... sch... constit... of hel... the in... the intra-chain bonds of the compound major helix into which they are finally twisted.

The kinds of inter-chain bonds that hold the primary helical unit of the collagen structure in that shape, and indeed go further and result in the end in a triad of such helices becoming also twisted round one another to form a coiled coil, are still under debate, though obviously some must be CO. . .NH hydrogen bonds and others may well be hydrogen bonds of the type postulated by

Gustavson (our much-respected doyen of the Collagen Group!) as linking the hydroxyproline OH group with a CO group on an adjacent chain. Incidentally, we must beware of perhaps misapplying Gustavson's idea, which is a deduction from the properties of *collagens* – collagens of different hydroxyproline contents. It is concluded that the bond confers resistance to thermal shrinkage and one might expect, therefore, to solution; but gelatin is soluble in warm water yet has the same hydroxyproline content and gives the same kind of large-angle X-ray diagram as collagen – the diagram, that is, from which the coiled-coil structure has been derived; and the earthworm's cuticle gives the same kind of diagram too, though, as first shown by Singleton (1955) in our laboratory at Leeds, it has the highest known hydroxyproline content (equivalent to 14.6 per cent of the total nitrogen) combined with the very low thermal-

would seem that the latter is the more plausible – without necessarily excluding the former entirely, nevertheless, because another, and contrasting, consideration not to forget is the fact that the
no
co

studies – and keen investigations to that end are being carried out these days in more than one laboratory, investigations to determine not merely the proportions of the amino-acid residues but also their order. Already such findings as, for example, the common occurrence of the sequence -prolyl-hydroxyprolyl-glycyl- make it seem

the structure under examination; for instance, more uniform and

plenty of scope (perhaps a little too much for present comfort) in what is now known of the molecular framework of the collagen group, including most if not all of the connective tissue fibres, to satisfy gradation and variability requirements. A great deal of

and I find that most inspiriting.

It follows that the main burden and responsibility of connective tissue research devolves more than ever on the procedures and results of *extraction*. The business of extraction and separation dominates biological chemistry and physics – it 'delivers the goods' – and here, in the needs of disentangling the connective tissue complex, we see a particularly fine illustration, in an exciting configurational setting too. Components are made available conforming in the first place to certain minimum structural specifications now partly recognizable, and these are then made use of, with such modifications as may be required and are permissible, in successive

medical science

improbably, a precursor of both, incorporated in an ideal or limiting edifice, such as a regular aggregation of the X-ray analyst's triple coiled coils The present incomplete and imperfect correlation between molecular form and chemical constitution is, however, symbolic and perhaps more representative of the actual state of affairs, in which the ideal plan, though governing in principle all the fibrous products, yet impresses itself to widely varying extents and, indeed, succeeds sometimes only so very indifferently as effectively to lose its identity.

Which brings me now, *via* this last remark, to a reconsideration o the apparently outstanding case of elastin – 'reconsideration', because I proposed as long ago as before the war that elastin might very well be a member of the collagen group but with a thermal contraction temperature below ordinary temperatures, and in the light of the chemical and structural attainments of the time we studied the matter by X-rays and obtained the first diffraction diagrams of genuine collagen-free elastin, unstretched and stretched (Astbury, 1938, 1940). More complete chemical evidence (see, for example, Tristram, 1953) has since made it clear that, though the glycine and proline contents of elastin are much the same as, if not even a little higher than, in orthodox collagen, there is a remarkable decrease in the proportions of the polar residues, not only basic and acidic but most noteworthy of all, hydroxyproline. We are therefore at once put in mind again of Gustavson's suggested inter-chain linkage between a peptide CO group and the hydroxyproline OH group, which reveals itself specially in its tendency to inhibit thermal contraction, and in this context elastin certainly looks like a direct extrapolation from collagen through lower and lower hydroxyproline contents. The effect of the loss of the stabilizing influence of the hydroxyproline linkages will be enhanced by the substitution of non-polar for most of the other polar side-chains besides, which will both eliminate more of the stronger cross-linkages and further lower the thermal contraction temperature by a process of 'internal plasticization', in the manner well known to high-polymer physical chemistry, where, in addition to the method of increasing plasticity (molecular mobility) by incorporating small *extraneous* molecules between the long chain-molecules, an impressive 'opening-up' can often be brought about by the artificial introduction of irregular side-chains in the main-chains themselves.

In the same connection one cannot help wondering about the relation of the configuration of polyglycine II to temperature. According to Meggy and Sikorski (1956), polyglycine has a transition temperature round about 60° C: when polyglycine I is dissolved in saturated calcium chloride solution then re-precipitated by addition of water, the precipitate at ordinary temperatures is polyglycine II, but between about 60° and 100° C it is a mixture of polyglycine I and II. The configuration is thus not directly dependent on the mode of chemical synthesis, but the fact that the helical form which is more stable at ordinary temperatures is the one simulated

and adapted in the construction of the collagen complex – that, and the occurrence of polyproline also in the same form, and that form [illegible]

tion' of the k-m-e-f group, whereas there is a striking emptiness [illegible]

in shape by *intra-chain* hydrogen bonds?

If only for these reasons, 'I think', to quote from my Procter Memorial Lecture (1940), 'that we still ought to work on the hypothesis that elastin is at least closely related to the collagen group'. But they are not the sole considerations: for instance, *à propos* of what I have just said about the X-ray evidence, was my phrase [illegible] finished, most stable, most 'normal', and best documented manifesta- [illegible]

may therefore be the normal, as it were, and immature collagen and elastin may be nothing like so different as they come to be in subsequent phases of growth.

And then, as intriguing a set of observations on possible collagen-elastin interrelationships as have been reported for a long time, there are the recent joint studies (Burton *et al.*, 1955) undertaken in the

University of Leeds by the Nuffield Gerontological Unit and the

structures as judged by appearances in the electron microscope and by histological staining tests; and what is more, when alkaline buffers are forced through pads of collagen, the percolates are found to be very rich in hydroxyproline and arginine, the two amino acids conspicuously deficient in elastin as compared with collagen. Were it not for this last finding one might be inclined to look askance at what seems to be shown in the electron microscope and by classical staining techniques, but all three kinds of results together make one think twice. Strictly speaking, all that the experiments demonstrate unambiguously as yet, is that the collagen complex can be broken down in such a way as to lead to preferential extraction of hydroxyproline and arginine – and this in itself is not unexpected, either, in view of the combined X-ray and chemical analytical indications discussed above – but let us hope they mean something far more gratifying. They must be followed up unremittingly, it goes without saying, and every effort made to prove, by X-ray and elasticity tests for example, that the man-made 'elastin', besides looking and staining like natural elastin, *is* the same sort of thing as natural elastin, as near as makes no matter.

All in all, it is not too bad a case for tentatively accepting elastin as a bona fide if somewhat surreptitious member of the collagen group, and I repeat that I personally like the idea.

The final answer goes back, of course, to the biosynthesis of the polypeptide chains and reactions about which we are abysmally ignorant. After these unknown initiating steps the evidence then points mainly to extracellular mechanisms, in the so-called ground substance; though indications are not lacking that the cell surface, if not still also the cell interior, plays its part too. The overall impression is of precipitation processes under more or less remote control – less say in the case of the crossed fibrils of the earthworm's cuticle fabricated on a layer of epithelial cells, and more say in the case of the threads ejected by the sea cucumber. The collagen group is truly 'of most excellent fancy'!

polysaccharides, and no talk on the collagen group and connective

orators imaginable: the nearer their job is to completion, the fewer the stoichiometric signs that they ever had anything to do with it. In the end they are rather like the Cheshire Cat – only their grin remains.

ON THE STRUCTURE AND FUNCTIONS OF THE MAST CELL

G. Asboe-Hansen

Mast cells are large connective tissue cells with an approximately central nucleus and ample cytoplasm with more or less densely arranged granules. Their shape and size show considerable variation. The granules are evenly distributed over the cytoplasm and stain metachromatically with certain basic dyes.

Histochemical, chemical and histophysiological studies have led to various theories regarding the function of the mast cells:

(1) In 1937 Holmgren and Wilander advanced the theory that the mast cells contain and form heparin. Jorpes, Werner and Åberg (1948), and Friberg, Graf and Åberg (1951) have demonstrated that the granular substance of the mast cells is not di- or tri-sulphated, but that it may be monosulphated, and may be the precursor of heparin.

(2) In 1950 I performed a series of experiments which led to the theory that mast cells may produce hyaluronic acid, perhaps by way of a heparin-like precursor and under some hormonal influence. It seemed obvious that heparin production could not be the only task of the enormous mass of mast cells in the body. Searching for the origin of the mucopolysaccharides of the connective tissue ground substance, I realized that the mast cell is the only connective tissue cell which has been shown to contain mucopolysaccharide material. Morphological and histophysiological studies compared with chemical data indicated that the material released contains hyaluronic acid.

(3) In 1953 Riley and West advanced the theory that the mast cells contain histamine which can be released to the tissues. Since then several authors have stressed the parallelism between the mast cell content of a tissue and its histamine value (Riley and West, 1953, 1955; Cass *et al*, 1954; Benditt *et al.*, unpublished). In 1955 Riley and his co-workers could not find conformity between the liberation of histamine and heparin in rats, though disruption and degranulation of the mast cells occurred in response to injection of the chemical histamine-liberator substance 48/80.

Hormones have been shown to influence the mast cells and the mucopolysaccharides of the connective tissue ground substance.

It should be emphasized, however, that the experiments and find-

tion might be expected to afford information of value in this discussion

I. STUDIES ON MAST CELL REACTIONS IN LIVING CONNECTIVE TISSUE OF THE HAMSTER CHEEK POUCH

These investigations were performed in collaboration with a Finnish colleague, Dr. Otto Wegelius, working in my laboratory. These experiments have led to a view which unites the different theories and assigns to the mast cells an acceptable position in relation to mucopolysaccharide as well as histamine. In addition they have afforded some information regarding the influence of hormones upon the cells.

The cheek pouch is drawn out, the upper layer carefully incised and the membrane stretched out by means of needles over a hole in a disc of cork. If desired this may be fitted into a moist chamber of

The thin membrane getting intact circulation was studied unstained by the phase contrast microscope, and, after staining, by bright field microscopy Toluidine blue in physiological saline, 1:100 to 1:100,000, was dropped on the exposed connective tissue surface of the membrane. The weak dilutions do not appear to be toxic to the mast cells, as degranulation and intracellular rearrangements of

The animals are anaesthetized by intraperitoneal injection of 0.08–0.15 ml. of a 6 per cent nembutal solution.

Hormones

The systemic treatment with various hormones was given in the form of injections into the femoral muscles, and the topical treatment as injections into the connective tissue between the two epithelial layers of the cheek pouch.

TABLE I

SYSTEMIC TREATMENT

(intramuscular injections)

Hormone	*Dose*	*Time of action*	*Results*
Corticotrophin	5+5 mg /day	7 days	Degranulation, clumping, vacuolation
Cortisone acetate	10 mg /day	5 days	Degranulation, clumping, vacuolation
Thyrotrophin	1 USP unit/day	5 days	Numerous well-granulated cells
Thyroxin	0 5 mg /day	5 days	Degranulation, small perivascular cells, intact
Somatotrophin	0 5 mg /day	20 days	Numerous well-granulated cells
Controls	Daily injection of physiological saline solution		

TOPICAL TREATMENT

(injections into the connective tissue of the extended cheek pouch)

Cortisone acetate	0 25 ml = 6 25 mg	38 hours	Degranulation, clumping, vacuolation
Hydrocortisone acetate	0 25 ml. = 2 5 mg *and* 0 5 ml = 5 mg	*5 hours and 38 hours*	Degranulation, clumping, vacuolation
Hydrocortisone tertiary-butyl-acetate	0 5 ml = 12 5 mg	38 hours	Degranulation, clumping, vacuolation
Controls	0 25–0 5 ml physiological saline sol or Tyrode sol		

The results were as follows:

Corticotrophin and *cortisone acetate* have been found to exert a[n] action on the mast cells in the cheek pouch after systemic treatmen[t] whilst cortisone acetate, *hydrocortisone acetate* and *hydrocortisone-tertiary-butyl-acetate* also act on topical injection. The cells become degranulated, the granules gathering into clumps, and vacuolation is not uncommon. After topical injection of cortisone acetate, hydrocortisone acetate, and hydrocortisone-TBA into the cheek pouch, the mast cells can no longer be identified in the immediate vicinity of the depot. Farther away mast cells are visible, but greatly changed showing large vacuoles and clumping of the granules (Fig. 4). A special phenomenon, viz. mast cells with large, round, ortho- or slightly metachromatic granules or drops, abound in the specimen.

Inspection of connective tissue after intramuscular *thyrotrophin* injections reveals well-granulated mast cells in large numbers.

Cheek pouches of animals treated with *thyroxin* showed a remarkable appearance. A considerable number of *small* mast cells were observed, primarily situated perivascularly. In the connective tissue, farther away from the vessels, mast cells were few and in various stages of degranulation. Clumping of the granules was quite a common phenomenon.

Following intramuscular injection of *somatotrophin*[1] the connective tissue exhibits large, well-granulated mast cells in large numbers, like the finding in animals treated with thyrotrophin

Control experiments were performed to ascertain the possible effect of the dye and manipulations.

We extended these investigations to include studies on mast cell responses to other agents in order to elucidate the functions of the cells.

Histamine

There exists much evidence that at least some mast cells contain considerable amounts of histamine; but the problem whether the mast cell changes observed after injection of histamine-liberator

[1]*Preparations*

Adrenocorticotrophic hormone = Acton, Frederiksberg Chemical Factories Ltd, Copenhagen.

Cortisone acetate = Cortone acetate, Merck & Co, Inc, New York, U S A.

Thyrotrophic hormone, the Armour Laboratories, Chicago, Ill, U S A.

Thyroxin, Hoffmann-La Roche & Co, Basel, Switzerland.

Hydrocortisone acetate = Hydrocortisat, Leo Pharmaceutical Products, Copenhagen.

Hydrocortisone-tertiary-butyl-acetate = Hydrocortone, TBA, Merck & Co, Inc, New York, U S A

Somatotrophic hormone, the Armour Laboratories, Chicago, Ill, U.S A.

substances are a specific response in some way bound up with the liberation of histamine, or a response to the tissue effects of the histamine liberated from other sources, is still unsolved.

The aim of the following experiments was, therefore, to study – under conditions as physiological as possible – the response of *living* mast cells to histamine as well as to histamine-liberators in connective tissue receiving unhindered blood supply and on the whole left largely intact.

The effects of distant injections of a neutral and isotonic solution of histamine hydrochloride, of compound 48/80 in physiological saline solution, of stilbamidine isethionate (M. & B.) in sterile water, of peptone (Witte) in physiological saline, and of sterile water were tested Stilbamidine is highly toxic; intracardiac injections were given very slowly in order to avoid immediate death. Compound 48/80[1] is a more specific histamine-liberator of lower toxicity. Water induces an osmotic imbalance and is found to liberate histamine locally (Fawcett, 1954).

Both cheek pouches of a hamster were examined, extended over two holes in a disc. As a control the left cheek pouch was examined before the distant injections of the mentioned agents. The connective tissue of the divided control pouch was stained for 5 minutes [illegible] Thereafter, [illegible] jected intra- [illegible] ncentrations

TABLE II

INFLUENCE OF HISTAMINE AND HISTAMINE-LIBERATOR SUBSTANCES ON LIVING TISSUE MAST CELLS

Agent	*Conc*	*Dose*	*Mast cell response*
Histamine HCl	1 mg /1 ml	0 5-2 mg	Degranulation, clumping
Cp 48/80	1 mg /1 ml	0 4 mg	Degranulation, clumping
Stilbamidine isethionate	10 mg /1 ml	7 5 mg	Degranulation, clumping
Peptone	100 mg /1 ml	50-100 mg	Degranulation, clumping
Sterile water		2 ml	Degranulation, clumping, disruption

Agents administered by intracardiac, intramuscular and intraperitoneal injections The observation periods were 3, 15, 30, 40 and 60 min.

[1] A polymeric condensation product of p-methoxyphenethyl-methylamine and formaldehyde, kindly supplied by Burroughs Wellcome & Co, England

The effects were studied in the right cheek pouch, handlec stained in exactly the same way as the control pouch.

A few minutes after the injections of histamine and histam liberators the connective tissue of the cheek pouches looked mor less oedematous.

Distinct, and often extremely marked degranulation of the m cells was observed following systemic administration of shock dos of histamine (Fig. 2) as well as of the histamine-liberators 48/8 and stilbamidine. The effect on the cells of compound 48/80 wa observed to set in within 5 minutes after the injection, and to reach a maximum within 30 minutes. Peptone induces slight degranulation, but never as marked as the above-mentioned agents. Following systemic administration of sterile water some cells were irreversibly disrupted, but most cells remained intact, though to some extent degranulated. Clumping of the granules was noticed in some of those cells which still retained their granules. There were occasional cells with apparently normal granulation (Fig. 1), or even groups of such cells, although almost exclusively around the larger blood vessels. By contrast, pronounced degranulation was always observed around the capillaries, small arterioles and venules. Velocity of the changes appeared to depend on dosage.

In the control experiments involving systemic administration of physiological saline and Tyrode solution similarly applied, and in the same amounts, degranulation was not more marked than in the directly stained specimens.

When studying mast cells visualized in the phase contrast microscope without staining, we were able to observe consistent changes in the mast cells after injection of histamine and histamine liberators. The cell contours became indistinct, and the granular structure was altered, rearrangement of the granules and vacuolation were observed, sometimes the pattern was entirely blurred. Apparently unaffected mast cells were also nearly always demonstrable by this method.

Serotonin (= 5-hydroxytryptamine)

Serotonin creatinine sulphate (National Biochemical Corp.) injected intraperitoneally into hamsters in doses of 10 to 20 μg. per gramme body weight induced pronounced oedema of the connective tissue. A considerable degranulation of the mast cells in the

substances are a specific response in some way bound up with the

f

living mast cells to histamine as well as to histamine-liberators in connective tissue receiving unhindered blood supply and on the whole left largely intact.

The effects of distant injections of a neutral and isotonic solution of histamine hydrochloride, of compound 48/80 in physiological saline solution, of stilbamidine isethionate (M. & B.) in sterile water, of peptone (Witte) in physiological saline, and of sterile water were tested. Stilbamidine is highly toxic; intracardiac injections were given very slowly in order to avoid immediate death Compound 48/80[1] is a more specific histamine-liberator of lower toxicity. Water induces an osmotic imbalance and is found to liberate histamine locally (Fawcett, 1954).

Both cheek pouches of a hamster were examined, extended over two holes in a disc. As a control the left cheek pouch was examined before the distant injections of the mentioned agents. The connective tissue of the divided control pouch was stained for 5 minutes ... Thereafter,

... jected intra-

... ncentrations

TABLE II

INFLUENCE OF HISTAMINE AND HISTAMINE-LIBERATOR SUBSTANCES ON LIVING TISSUE MAST CELLS

Agent	*Conc*	*Dose*	*Mast cell response*
Histamine HCl	1 mg /1 ml	0 5-2 mg	Degranulation, clumping
Cp 48/80	1 mg /1 ml	0 4 mg	Degranulation, clumping
Stilbamidine isethionate	10 mg /1 ml	7 5 mg	Degranulation, clumping
Peptone	100 mg /1 ml	50-100 mg	Degranulation, clumping
Sterile water		2 ml.	Degranulation, clumping, disruption

Agents administered by intracardiac, intramuscular and intraperitoneal injections. The observation periods were 3, 15, 30, 40 and 60 min

[1] A polymeric condensation product of p-methoxyphenethyl-methylamine and formaldehyde, kindly supplied by Burroughs Wellcome & Co , England.

The effects were studied in the right cheek pouch, handled and stained in exactly the same way as the control pouch.

A few minutes after the injections of histamine and histamine-liberators the connective tissue of the cheek pouches looked more or less oedematous.

Distinct, and often extremely marked degranulation of the mast cells was observed following systemic administration of shock doses of histamine (Fig. 2) as well as of the histamine-liberators 48/80 and stilbamidine. The effect on the cells of compound 48/80 was observed to set in within 5 minutes after the injection, and to reach a maximum within 30 minutes. Peptone induces slight degranulation, but never as marked as the above-mentioned agents. Following systemic administration of sterile water some cells were irreversibly disrupted, but most cells remained intact, though to some extent degranulated. Clumping of the granules was noticed in some of those cells which still retained their granules. There were occasional cells with apparently normal granulation (Fig. 1), or even groups of such cells, although almost exclusively around the larger blood vessels. By contrast, pronounced degranulation was always observed around the capillaries, small arterioles and venules. Velocity of the changes appeared to depend on dosage.

In the control experiments involving systemic administration of physiological saline and Tyrode solution similarly applied, and in the same amounts, degranulation was not more marked than in the directly stained specimens.

When studying mast cells visualized in the phase contrast microscope without staining, we were able to observe consistent changes in the mast cells after injection of histamine and histamine liberators. The cell contours became indistinct, and the granular structure was altered, rearrangement of the granules and vacuolation were observed; sometimes the pattern was entirely blurred. Apparently unaffected mast cells were also nearly always demonstrable by this method.

Serotonin (= 5-hydroxytryptamine)

Serotonin creatinine sulphate (National Biochemical Corp.) injected intraperitoneally into hamsters in doses of 10 to 20 µg. per gramme body weight induced pronounced oedema of the connective tissue. A considerable degranulation of the mast cells in the

cheek pouches was observed within 10-30 minutes. Irreparable breakdown of the cells, was observed only in exceptional cases.

Mast Cells and Tissue Water

The following series of experiments is concerned with the *local* effect of various fluids upon connective tissue mast cells. One of the two cheek pouches of the hamster was invariably used for control. Sterile water, physiological saline solution, Tyrode solution, 2 per cent saline solution, solutions of hyaluronidase and of hyaluronic acid were injected between the two layers of the cheek pouch or dropped on to the lower membrane of the outspread split pouch. In order to approach physiological conditions as far as possible, the hamsters were placed on a warming stage mounted on the microscope. One of the hyaluronidase solutions used contained 100 viscosity reducing units per ml. and another, highly concentrated

TABLE III

INFLUENCE OF VARIOUS FLUIDS ON LIVING TISSUE MAST CELLS

Fluid	*Changes in mast cells within 30 min*
Sterile water	Degranulation, disruption, granule swelling
Physiological saline	Degranulation
Tyrode sol.	Degranulation
2 per cent saline sol.	Shrinkage
Hyaluronidase in physiological saline (100 and 1000 VRU/ml.)*	Rapid degranulation
Hyaluronidase in Tyrode sol. (100 and 1000 VRU/ml.)	Rapid degranulation
Hyaluronic acid 1 and 2 per cent in physiological saline	Insignificant changes
Traumatization (tissue edema)	Degranulation, disruption, granule clumping

* *Viscosity reducing units*

one 1000 V.R.U./ml., the last mentioned preparation contained 3000 V.R.U./mg., or about 27,000 V.R.U./mg.N.[1]

Hyaluronic acid, produced from bovine vitreous humour and from human umbilical cord[1] was applied – in 1 per cent and 2 per cent solutions.

In order to obtain a local tissue oedema by mechanical trauma the cheek pouch was rubbed a few times between the thumb and the index finger.

Injection of the fluids into the connective tissue of the cheek pouches invariably caused disruption, i.e. irreversible damage, of some mast cells. Perceptibly fewer disintegrated cells were seen after the dropping procedure.

After application of water a considerably larger numbe
in various stages of degranulat
stained control pouches.
swollen. Many granules ... free in the connective tissue ground substance after disruption of the cells, still showing intense metachromasia; they retained their colour unchange
hours. Varying numbers of
plasm metachromatic gran
was interpreted as eviden ... mast cell granules. These granules ... their metachromasia for some time within the bodies of the phagocytizing cells (Fig. 3).

After treatment with physiological saline and Tyrode solution a considerable increase in the number of degranulated cells was observed as early as 20-30 minutes after application. These cells were fairly often surrounded by a zone of intercellular substance showing more intense metachromasia than more distant areas. Entirely degranulated cells showed a basic pattern like a honeycomb or sponge (cf. Fig. 2).

Thirty
ma
trea
gra

in
gra ... a few cells were well
stai ... metachromatic after a fairly long time of treatment.

[1] Kindly

These cells may have been protected by adipose tissue, thicker fibrous layers, etc. The granules remaining in partially degranulated cells showed normal metachromasia. The size of the cells was not definitely altered. No extracellular metachromasia was noticed,

(cf. Fig. 1). Later on a slow degranulation took place.

After mechanical traumatization of a cheek pouch mast cells with

macrophages with phagocytized granules.

DISCUSSION

It is obvious that the granule substance may come into the ground substance, either by a *disruption* caused by some violent – physical or chemical – action during which the cell perishes, or by a physiological *degranulation*, probably involving some alteration of the granule substance, e.g. with regard to water solubility, before or during the passage from within to outside the cell.

Studies of living connective tissue of the hamster under the influence of hormones revealed morphological changes of the mast cells, largely identical with those seen in dead, fixed tissues of other animals and humans similarly treated. It is evident that the extremely marked and consistent changes observed must reflect an interference with the functions of the mast cells.

The degranulation of the mast cells invariably seen after administration of histamine and histamine-liberators shows that the mast cells of the hamster are sensitive to the influence of both. The observation that histamine exerts an action as powerful as that of the histamine-liberators, indicates that the mast cell changes are an

than a

eleased;

bstance

must also be released. Whether degranulation, which is a function of the living cell, entails a release of histamine together with the metachromatic substance, still remains an open question.

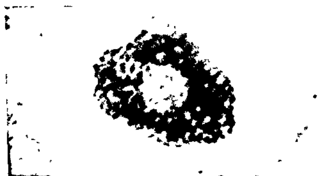


Fig 1
Normal mast cell loaded with metachromatic granules Staining toluidine blue in physiological saline 1 1000 Magnification ×2,200

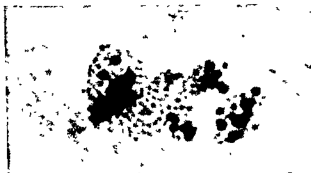


Fig 2
Mast cell showing partial degranulation after intraperitoneal injection of histamine Notice the basic structure simulating a sponge or a honeycomb Staining toluidine blue in physiological saline 1 1000 Magnification ×2,200

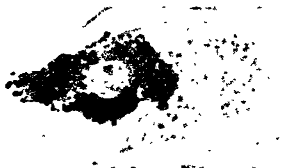


Fig 3
A mast cell during disruption and a macrophage containing some metachromatic granules Staining toluidine blue in physiological saline 1 1000 Magnification ×2,200



Fig 4
Mast cells influenced by hydrocortisone acetate The cells are vacuolated and partly degranulated the granules are clumped together into irregular masses Staining toluidine blue in physiological saline 1 10,000 Magnification × 1,500
(*Acta endocrinol*, 22, 157, 1956)



Fig 5
Two atretic follicles (rabbit ovary) surrounded by mast cells Staining toluidine blue in 40 per cent ethyl alcohol 1 1000



Fig 6
Dermographic stripe in the skin of a patient suffering from urticaria pigmentosa

The disruption following upon application of water is probably due to osmosis.

The rapid release of granules from the mast cells after local application of hyaluronidase was interpreted as a compensatory reaction to the topical situation established by a breakdown of the hyaluronic acid of the ground substance, and an increase in the diffusivity of water in the tissues. It is noteworthy that the cells are insignificantly or very slowly altered by the influence of a hyaluronic-acid-containing Tyrode solution. The last-mentioned fluid is possibly the real control fluid affording a physiological environment for the mast cells

A common denominator of the effects elicited by histamine, histamine-liberators, serotonin, water, physiological saline, Tyrode's solution and hyaluronidase is the increased water content of the environments of the mast cells. Histamine increases capillary permeability, and histamine-liberators are supposed to cause a tissue oedema of a similar kind and genesis.

The implications of degranulation are not fully understood. The present observations, however, indicate that a mast-granule release begins to change free water in the tissues into a mucinous fluid, a sort of connective tissue ground substance. Any disturbance in the physico-chemical balance between the intra- and the extracellular substance may immediately elicit a mast cell response.

The perivascular situation of many mast cells reminds one that they may to some extent be functionally linked to vascular permeability, being ever ready to change a perivascular oedema into mucinous ground substance.

The connective tissue formation caused by carcinoids and their metastases (Hedinger and Gloor, 1954) might possibly be related to and started by the degranulating effect of serotonin, unceasingly released, upon the mast cells. The tissue oedema induced by this agent could be primary to an organization process.

It is my impression that the cortisone group of steroids exerts a cytotoxic effect on the mast cells. It gives rise to other changes besides degranulation. Thyrotrophin and growth hormone appear to stimulate the mucinous system, and the formation of mucopolysaccharide seems to be primary in relation to the binding of water in the tissues.

It is my opinion that the mast cell release of granule substance may be *active* as a consequence of hormonal stimulation, or *passive* as a

consequence of an increased content of free water in the connective tissue ground substance.

II. BASOPHIL LEUKOCYTES AND TISSUE MAST CELLS

The finding by Graham and her associates (1955) that the basophils of the blood contain relatively large amounts of histamine seemed extraordinarily interesting in view of the current discussion as to whether mast cells and basophil leukocytes are the same type of cell or carry out similar functions. Staining of the cytoplasmic granules corresponds to the staining of the granules of the tissue mast cells; they are metachromatic, coarse and of a size like those of the tissue mast cells. The basophil leukocytes are somewhat smaller than the mast cells of connective tissue. The nucleus of a basophil leukocyte is fragmented, usually bisegmented, whereas that of a tissue mast cell is generally unsegmented. The basophils are formed in the bone marrow, whereas the tissue mast cells are believed to be recruited from perivascular mesenchymal cells. Mast cells have never with certainty been observed to pass through vascular walls, but they are at times seen among endothelial cells, and they are

release histamine?

The number of basophil leukocytes has been reported to decrease in individuals under the influence of cortisone, like mast cells (Code *et al.*, 1954).

In rabbits' ovaries the following findings have been made: 9-18

which we are at present studying in more detail. In the histological sections the blood vessels of the ovaries and tubes were densely packed with basophil leukocytes or mast cells; there were cells with

rabbits has so far revealed an abrupt drop and a subsequent, even increase. This is remarkable considering that mast cells are scarcely seen in non-stimulated rabbit ovaries. At the moment Dr. Boué of our group is studying the variations in these cells and their relation to the tissue mast cells.

The presence of mast cells or basophils in stimulated ovaries might indicate a task in the repair process following ovulation or in the organization of an oedema. Whether the phenomenon may have any correlation to the presence of hyaluronic acid reported to be present in the cumulus of the egg is an open question.

I would like to supply one further argument in this discussion: a patient with generalized urticaria pigmentosa (Fig. 6). He proved to have up to 10.3 per cent mast cells in the bone marrow (normally less than $\frac{1}{2}$ per thousand), up to 9.5 per cent basophil leukocytes in the blood (normally less than $\frac{1}{2}$ per cent) (Table IV) and in his rather

TABLE IV

White Blood Pictures

	July 1955	July 1955	Sept. 1955	Oct 1955	Jan 1956	Feb 1956	Apr 1956
W B C	5,600	7,000	7,700	8,000	8,000	10,000	7,600
Differential count, %							
Rod shapes	2 0	2	1 5	4 5		1 5	2 0
Segmental	42 5	53	62.0	62 0	57	62 0	60 0
Eosinophils	3 5	7	4 5	4 0	6	4 5	2 5
Basophils	9 5	5	3 0	4 0	5	3 0	7 5
Lymphocytes	37 0	29	25 0	21 5	30	25 0	20 5
Monocytes	2 5	4	4 0	4 0	2	4 0	7 5

enlarged liver there were enormous quantities of mast cells. In addition, he had disseminated xanthomata. He did not show any signs of heparinaemia, and the heparin-resistance, protamine-titration and thrombin-generation tests were negative. Clotting time, also after rubbing of the skin, was normal. He did not have attacks of flushing, but itching was a predominant symptom. The blood lipid values were within the limits of normal, but the cholesterol values were relatively high.

This case gives some support to the assumption that basophil leukocytes and tissue mast cells are the same type of cell, but it must be pointed out that the case is unique.

GROUP DISCUSSION

DR SNELLMAN wondered what evidence existed that heparin was a precursor of hyaluronic acid. He felt it was unlikely that such a complicated chemical change would occur *in vivo*. He said that there was no

hyaluronic acid in liver capsule in spite of mast cells DR. ASBOE-HANSEN said that heparin and hyaluronic acid were both acid mucopolysaccharides The mast cell was the only connective tissue cell known to contain mucopolysaccharide substances. The metachromatic material which, [illegible] substance was sensitive to [illegible]

[illegible]

if the mast cell content was high. There was no doubt that these cells had several functions It should be pointed out also that there were chemical differences between the mast cells of different species and between mast cells in different locations of the same individual Intra- [illegible]

[illegible] rat and human skin, men- [illegible]

DR ASBOE-HANSEN could not be persuaded that the problem could be solved by tissue culture experiments One might have granulated mast [illegible] the last possibility must certainly be [illegible]

cells involved ideal fixation, preparation and histochemistry, and perhaps isotope studies as well

DR. MEYER pointed out the wide variety of unrelated polysaccharides [illegible] type of polysaccharide, together with a [illegible] cursor of heparin. [illegible] his monosulphated ester. No one had yet isolated a sulphated mucopolysaccharide from synovial fluid, although there was a very small quantity of sulphate. The only

polysaccharide isolated from synovial fluid was hyaluronic acid, which of course, is not sulphated.

Dr. Asboe-Hansen said that mast cells may contain several polysaccharides in several stages of polymerization and sulphation. Synovial membrane has been found to contain a lot of degranulated mast cells near the cavity and heavily granulated ones deeper down The metachromasia of the amorphous ground substance varied inversely with the intracellular metachromasia

In answer to several points raised by Dr Neuberger, Dr. Asboe-Hansen said the life span of mast cells was unknown. Mitoses were scanty even in very large accumulations of mast cells seen in experimental tumours in mice. The mast cells appeared to regenerate from the peri-

necessarily all be in the same stage of physiological granulation or metabolism and consequently the cells may differ in uptake of isotopes He felt that Dr Asboe-Hansen's illustrated accumulations of mast cells in ovarian sections may not have been derived from the blood, but may

chromatic material, regularly encountered in such necrotic areas, was necessarily derived from the mast cells In fact the 'mast cells' in these circumstances were perhaps phagocytes loaded with ingested metachromatic material, suggesting that one type of mast cell may arise in this way.

Dr. Baló asked if there were any experiments on the phagocytosis of heparin He recalled that Walton, in 1954, claimed heparin could not be

Dr. Asboe-Hansen agreed that, in some instances, it might be difficult to differentiate between macrophages and mast cells but he believed it was possible in living tissues because of the background structure of the mast cells. After cells were disrupted, metachromatic granules were found in both the ground substance and the cytoplasm of the phagocytes After provoking degranulation of the mast cell, a metachromatic amorphous material appeared around the altered mast cells, except in hyalu- [illegible]

ON THE TOPOGRAPHICAL CYTOCHEMISTRY OF TISSUE MAST CELLS

B. Sylvén

Since the time of Ehrlich it has been widely accepted that the mast cell granules present metachromasia and also show basophilia to basic aniline dyes. This contention further strengthened by the results of Jorpes (1935), Holmgren and Wilander (1937) and Wilander (1938) led to the opinion that the mast cell granules *per se* contain and even 'are' heparin as stated by Jorpes (1946). Subsequently more detailed investigations on the cytoplasmic constituents of tissue mast cells by means of the freeze-vacuum dehydration technique and carefully controlled differential centrifugation methods led to the view that the native heparin material was located most likely as part of the microsomal substance within the intergranular compartment, thus surrounding the basophilic large granules.

Since I am mainly responsible for the latter interpretation you may allow me to discuss and evaluate the evidence in favour of these interpretations, and also to try to reconcile the various views with more recent data on the ultrastructure of mast cell cytoplasm. Let me first say that this question is no great matter of dispute, rather a question of academic interest. It is a matter of small distances, about 100 or a few hundred Å units, which, however, may be of some importance when function and metabolism are placed on more secure grounds. The sub-miscroscopic dimensions of mast cell components would then seem suitable for cell fractionation studies combined with chemical and staining assays of fractions obtained.

The old contention is based on a large accumulation of staining results routinely performed on tissues pretreated with various fixatives. In tissue sections of a thickness between 5 to 10 or more microns, large mast granules are seen, having a diameter in general between 200 to 500 millimicrons. The large granules were found to exhibit different degrees of metachromasia, some apparently red stained, others in tones of bluish-red, distinctly violet, and bluish or bluish-black. Very little intergranular cytoplasm was seen, and

those who have described this material by using other stain methods quite strictly state that 'the intergranular cytoplasm clear and homogeneous (*oxyphilic!*)' (Michels, 1928, cit. from p. 25. Little attention was paid to other staining methods. It would worth while to mention that the granules generally take up hema toxylin and that some large granules appear to be stained also with eosin and orange G (Lehner, 1924).

The granules further show a higher degree of refractivity than the rest of the cytoplasm, presumably due to a larger mass content, which facilitates their identification in unstained samples. A number of more specific staining reactions suggest that the granules may contain glycogen, protein, lipid, possibly RNP and some further Schiff-positive carbohydrate(s) or lipid material and a variety of enzymes.

The staining results would thus seem to suggest that the mast granules contain a number of different non-classified component Their marked basophilia has to be explained by the presence of som acid material, which does not seem to be heparin but possibly nucleic acid or some acid polysaccharide. So far, however, the inter-granular cytoplasmic materials have not been considered in detail.

At this point I would like to mention the position of the problem around 1940 in the laboratory of Hjalmar Holmgren. Together with Jorpes and Wilander he found evidence that mast cells contained heparin and was studying the metachromasia of mast cell granules, their morphology, histogenesis, etc. However, he was worried about the variations in staining characteristics noted in many mast cells; some granules were not metachromatic but instead strongly basophilic and stained blue to bluish-black. This was then believed to signify different functional stages, i.e. suggesting that some granules in the course of heparin production had not yet fulfilled the complete synthesis. Holmgren was quite convinced that the granules were not made up of pure heparin, but believed that heparin was a fair part of the granular mass. Incidentally, little attention was then paid to the new approaches in cytochemistry developed by R. R. Bensley, N. L. Hoerr, A. Claude and others. At that time, we almost forgot the possibility that heparin might be located in the seldom observed amorphous intergranular substance.

Now, my interest was aroused by histological studies of mast cells. Most aqueous and other fixatives recommended for the effective precipitation of metachromatic material were found to involve

cells were concerned. In thin fresh-frozen and dried sections purely basophilic and non-metachromatic mast cell granules were often seen Pictures of mast cells, like those published (Julén, Snellman and Sylvén, 1950), indicate that the 'amorphous' intergranular substance (cf. Sylvén, 1950) stained *metachromatic and oxyphilic.* Great caution was further justified in interpreting the staining reactions of cytoplasmic granules with dimensions below or very close to the lateral and axial resolving powers of the light microscope. Other observations suggested that metachromatic material was easily dissolved or could be

the course of carcinogenesis experiments.

It then seemed necessary to apply chemical fractionation methods in order to obtain more detailed information on the cytochemistry of these cells. At this stage, co-operation was obtained with Dr. O.

First, a mild method suitable for the extraction of the native heparin complex was found by using thiocyanate solutions, which present a unique capacity to bring the metachromatic mast cell material ... is material was ... isophilia of the ... bound to the granules or the possible union was by very weak bonds only.

found ... still ... mater

aggregate and later disintegrate in more acid media. We found that the microsomal mast cell material disperses only around pH 7, below pH 6 there is an increasing tendency to flocculation. An increase in acidity is thus most deleterious since the microsomal

material precipitates on the surface of the large granules and becomes also very firmly aggregated to all sorts of other cell materials in suspension. This was a real catch in the fractionation technique and *seemed further to explain part of the artifacts produced by the histo*logical preparation methods (cf. below).

If now the pH was kept at 7 during the whole fractionation procedure

washe

nate.

(cf. Novikoff *et al.*, 1952). The first large granules concentrate contained also some microsomal material, and the supernate contained some very fine particles which could not be sedimented by centrifugation at 60,000 g. The bulk of heparin material was part of the supernate as a sub-microscopic particulate matter with diameters below 10 millimicrons. The final supernate contained four different non-sedimentable fractions distinguished by electrophoretic mobility, and only one of these fractions (called u_4) contained heparin (Snellman, Sylvén and Julén, 1951). This native heparin material was found to be a heparin-lipo-protein complex with a particle size of about 100,000 and an electrophoretic mobility of 5.9×10^{-5} sq. cm./volt/sec. Purified heparin shows under similar conditions a particle size of about 17,000 and a mobility of $18-22 \times 10^{-5}$ sq. cm/volt/sec.

All fractions obtained during the course of fractionation and all

ctions showing metachromasia

the final supernate. *Washed large granules were free of heparin.*

The sub-microscopic particulate matter in the final supernate as well as part of the sedimentable microsome mass showed a marked *oxyphilia*, which we ascribe to the presence of basic protein, contained in any one of the two or three additional protein components devoid of heparin. The oxyphilia was of the same order as that seen in freeze-vacuum dehydrated mast cell cytoplasm. Both the sedimentable and non-sedimentable microsomal material showed a similar colloidal behaviour to various hydrogen ion concentrations as other microsomes have done Further, neither the large granules, nor the microsomes presented a very marked solubility in the native state as long as the pH was kept at 7. Heparin was not dissolved from the native complex even when the suspensions were kept for days.

Later on, Hedbom and Snellman (1955) isolated and analysed the

washed non-metachromatic large granules concentrate. Electron micrographs showed well-preserved large mast granules with diameters between 200 to 500 millimicrons. Ultra-thin sections have not yet been made. The general composition of the granules was: protein 72 per cent, lipids 24 per cent, RNA 3.6 per cent and some small amounts of carbohydrates and histamine. Some enzyme activities were demonstrated. The amount of heparin was less than 0 3 microg. per gramme of washed large granules. *Practically no trace of heparin was thus found*; traces of hexuronic acid were probably present. Thus, the composition conforms well with the general composition of other mitochondria and large secretory granules.

Our joint results led to the suggestion that the native heparin complex was very likely housed in the intergranular cytoplasm of mast cells as part of the microsomal material. A tentative reconstruction of the possible sub-microscopic organization of mast cell cytoplasm was made on this basis, and it was emphasized that our interpretation would explain the variable metachromasia of mast granules. With reference to the marked basophilia of mast granules we may point to the high content of RNA and/or other protein or lipid components, but we did not find a large amount of acid polysaccharide material as had been expected. Whether some smallish amount of a carbohydrate heparin precursor or other ground substance polysaccharides is present in the granules remains to be investigated. The granules appear to contain a trace of a non-metachromatic polysaccharide material devoid of anti-clotting effects but its nature has not yet been investigated. Thus from chemical and structural points of view it seems to us very unlikely that heparin is a precursor to hyaluronic acid, or that the latter is convertible into heparin.

We have so far no topical knowledge of the enzyme distribution and cannot suggest where the different steps of heparin synthesis may take place. To our mind it seems, however, plausible that the synthesis occurs in the ground substance of the cytoplasm in close co-operation with the large granules, perhaps in such a way that the final esterification is performed near the surface of the large granules.

Our findings and interpretations have led to different comments and a certain amount of confusion is noted in later reports (Zollinger, 1950; Friberg, Graf and Åberg, 1951; Wislocki and Fawcett, 1951; Montagna, Eisen and Goldman, 1954). However, it does not seem that the usual histological techniques, phase-contrast studies and the

inspection of thick whole-mounts of mast cells have progressed sufficiently to allow definite conclusions to be drawn from studies undertaken at the limit of or below the resolving power of the light microscope. Moreover, the use of acid solutions as fixatives (Helly's fluid – pH about 3.3 – used by Montagna *et al.*, 1954) and/or during staining will undoubtedly precipitate the mast cell intergranular microsome material in the same way as in *in vitro* experiments. The intergranular material following denaturation will unfortunately precipitate and stick to the surface of the large granules as well as to other elements, which thus will acquire a metachromatic shade.

From phase-contrast studies, Zollinger (1950) claimed that the granules had a water-insoluble protein skeleton and somehow inside this the metachromatic heparin component would be harboured. It is, however, difficult to accept this speculation when the granules so easily can be prepared in an apparently undamaged state (cf. Hedbom and Snellman, 1955) and are shown to be almost devoid of heparin (cf. above).

Köksal (1953) claimed to have obtained some metachromatic mast granules by shaking tissue spreads in distilled water for 1-2 hours without pH control. It seems clear that he obtained mast granules surrounded by a coating of metachromatic material. On the contrary, Zollinger found that mast cell granules treated with water released their metachromatic material, became orthochromatic and then lysed.

Some investigators have expressed some doubt as to our observation that the supernate fraction, containing – among other things – the native heparin complex, is oxyphilic and not basophilic. This may, however, be explained by the presence of oxyphilic protein material together with the native heparin complex. It seems quite evident from osmotic and other considerations that heparin in the native state cannot exist as a dissociated polyelectrolyte. We have said that it seems to be combined with a lipoprotein moiety, which may stain with eosin, and that there are other microsomal components of cytoplasm which may show eosinophilia. The heparin complex as such has enough electronegative surface charges available for showing metachromasia (cf. Sylvén, 1955).

Another question has to be considered if in our fractionation experiments we overlooked a dissolution effect leading to a very rapid and early release of heparin when the tissue mast cells are suspended in phosphate buffer. This would be an objection common

to differential fractionation procedures as such performed in aqueous solutions (cf. Novikoff *et al.*, 1952). Generally speaking, if a water-soluble substance forms part of any granular fraction, some of it may be found in the supernate. But as far as we know, no one has attributed a substance solely occurring in the supernate and not at all recovered in the granular fraction as belonging to the granules. Thus, our mast cell fractionation results seem only explainable on the assumption that the native heparin complex occurs outside the granular membranes.

This is as far as present evidence goes. We all hope that further details of the fundamental cytochemistry of mast cells will be revealed leading to a closer understanding of the physiology of these peculiar cells. The important discovery of histamine in mast cells (Riley and West, 1953), recently corroborated by chemical extractions (Hedbom and Snellman, 1955), lends further support to the assumption that connective tissue mast cells take part in local tissue reactions of various kinds.

GROUP DISCUSSION

DR NEUBERGER said that the only possible criticism against Dr. Sylvén's interpretation of his data was the very remote possibility of loss of heparin from the granules during his isolation process. If the molecular weight of the native heparin-protein complex were high, this possibility would be unlikely. If it were only 15,000 or less, it might easily leak out.

DR. SYLVÉN replied that the isolated heparin particle size was about 17,000, but under similar experimental conditions the native heparin-protein complex had a particle size (molecular weight) of about 100,000.

DR SNELLMAN felt that the electron microscopic pictures showing the granules as undamaged round balls would make it highly improbable that the heparin complex had leaked out from the granules during the isolation processes

DR GILLMAN suggested that heparin may be intimately connected with the minerals and their movements (especially calcium) in the mast cells and elsewhere Heparin also seems to exert some lipaemia clearing role not only in the circulation but also *in vitro* The presence of calcium ions seems to be necessary for the breaking of the chylomicron emulsion with

same time. Comparison of the specific radioactivities (in terms of nitrogen) showed that plasma in skin amounted to less than 1 ml. per 100 gm. skin. If we assume that after five minutes the iodine-labelled albumin had completely mixed with the intravascular albumin, but had not penetrated to a significant extent into the extravascular space, it can be concluded that the amount of blood present per 100 gm. skin is less than 1.5 ml Haemoglobin estimation of the skin sample indicated that the amount of blood was of the order of 0 4 ml. per 100 gm. Thus the two methods, whilst not giving good agreement, suggest that the blood present in skin cannot be more than 1.5 ml. per 100 gm. The results to be discussed later showed that the total amount of albumin which can be isolated from skin is at least 10 or 15 times greater than the maximum amount which can be present within the blood vessels.

Rate of equilibration between plasma and skin. Iodine-labelled plasma proteins were injected into two rabbits and one of the animals was killed after 4 days, while the other was killed after 10 days. The radioactivity in the plasma was measured and also that of successive extracts of skin. When the radioactivities were compared on a nitrogen basis, successive extracts of the four-day experiments showed that the skin extracts had between 23 and 32 per cent of the plasma activities. In the ten-day experiment the average value of five skin extracts was 50 per cent of that of plasma, again expressed on a nitrogen basis. It appeared possible that these differences were due to a variation between animals and a series of experiments was therefore done in which iodine-labelled albumin, carbon-labelled albumin, carbon-labelled antibody and a foreign protein, horse serum, were injected in various combinations into ten rabbits. The

per 100 gm. of skin, and the lowest values were observed in the one experiment when the animal was killed 8 hours after injection. The values between 2 and 8 days varied somewhat, but were equivalent to figures ranging between 4 and 8 ml. of serum per 100 gm. of skin (Fig. 1). It appears that a steady state, which possibly indicates complete equilibration between plasma and skin, is reached in about 8 days. It seems to require about 3-4 days to get about half the skin albumin replaced by plasma albumin. But in view of the marked variation between animals these values are only approximate. In

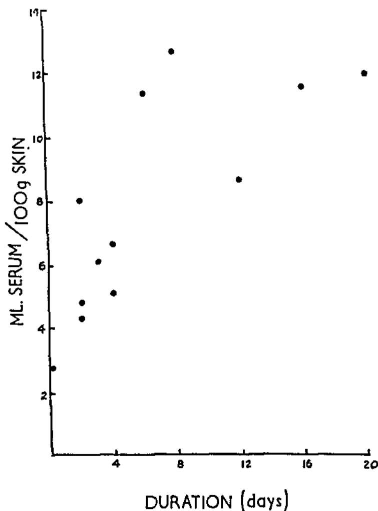


Fig 1

The changes of radioactivity of skin albumin with time

The radioactivity of skin extracts were compared on a nitrogen basis with that of plasma albumin obtained from plasma at the same time The results were calculated in terms of ml of serum per 100 gm. skin.

one experiment carbon labelling and iodine labelling gave similar results suggesting that the latter method is not likely to yield misleading information.

Heterogeneity of the skin albumin fraction. The experiments discussed above indicated that either complete equilibration between the skin and plasma is very slow or that the fraction which is designated albumin *consists not only of serum albumin but also contains* another protein, possibly formed in the skin, having a solubility characteristic of albumin. [^{14}C] Glycine was therefore injected into a rabbit and the animal was killed 8 hours after injection. The albumin fraction from skin was fractionated by column electrophoresis (Porath, 1956). Serum obtained from this animal was also subjected to fractionation by the same technique. Skin extracts gave a fraction designated I with a specific activity of 414 counts/min./mg. carbon. This material was examined serologically with an anti-rabbit albumin serum obtained from a goat. Quantitative precipitative reaction was carried out both on Fraction I of skin extract and on two preparations of rabbit serum albumin. Fraction I had about 87 per cent of the precipitating power of that of authentic serum albumin indicating that it consisted of approximately 87 per cent pure albumin. A second fraction obtained by column electrophoresis of

[illegible]

logically at least not equivalent to Fraction I. It contained some material capable of reacting or cross-reacting with anti-rabbit
albumin, as well a [illegible]
antibody in the ant [illegible]
material behaving [illegible]
fraction obtained [illegible]
had a still higher radioactivity, i.e. 957 counts/min./mg. It was therefore concluded that the skin extract which represented an albumin fraction as defined by solubility and electrophoresis contained material amounting to probably about 60 per cent of the total protein, which was immunologically identical with serum albumin but it also contained at least two other components. The high radioactivity of the fractions containing predominantly material other than serum albumin is compatible with the assumption, but does not prove that this other protein is made in the skin from the radioactive glycine supplied.

DISCUSSION

A large proportion, probably about 95 per cent of that part of the water-soluble protein fraction of skin which is not precipitated by 26 per cent sodium sulphate, resembles in its electrophoretic behaviour and general properties plasma serum albumin, but the fact that its specific activity, in the experiments in which iodine-labelled albumin was injected, never exceeded 65 per cent of that found at the same time in the circulating albumin indicates that the skin fraction designated as albumin was not pure. Indeed the results of the fractionation with the Porath column showed that this material contained at least two fractions, one closely resembling serum albumin and another fraction which may itself be heterogeneous and which consisted probably mainly of a protein of albumin character, possibly produced locally by cells in the skin. Our data do not allow an accurate estimation to be made of the relative proportion of serum albumin in the albumin fraction obtained from skin, but the isotope and immunological data suggest that serum albumin may constitute about 60 per cent of the total albumin.

The claim of identity of this material with plasma serum albumin is based on its serological behaviour, electrophoretic mobility, its optical rotation under two sets of conditions and finally its ultra-violet absorption spectrum in acid and alkali. The chemical and physical criteria used are by no means conclusive, especially since the amino-acid composition of the various fractions was not determined, but the immunological criterion is believed to be quite decisive. In addition, the results of the various isotope experiments indicate beyond any reasonable doubt that a large part of the soluble protein consists of serum albumin.

The evidence with regard to the other plasma protein fractions is less conclusive. Electrophoretic evidence has been obtained suggesting the presence of all three globulin fractions but in most of our experiments peaks were not as clearly defined as might be desired and there were strong indications of the presence of significant amounts of other proteins. There is thus little doubt that skin contains serum globulins in addition to serum albumin, but possibly not in the same proportions as in the blood.

Total amount of serum protein in skin. The data represented showed considerable scatter between different animals and thus and the lack of complete purity of our fractions make it impossible to produce a

very accurate estimate of the amount of albumin present in rabbit skin. But the data, particularly the results obtained by the use of iodine-labelled albumin, suggest that 100 gm. of skin may contain plasma protein equivalent to that present in approximately 12-16 ml. of plasma. This is equivalent to a protein content of about 1 gm. of plasma protein per 100 gm. of skin. If it is assumed that about 60 per cent of the total space of skin is extracellular, and plasma proteins are confined to the extracellular space, this would mean that the concentration of plasma proteins in the extracellular compartment of skin is about 1.5 to 2 per cent. This value is similar to that obtained for plasma protein in lymph from the limbs and thus is likely to be a reasonable estimate. On the basis of this rather rough estimate it would appear likely that the total amount of plasma protein in the skin is equivalent in amount to about 30 per cent of that present in the circulating blood. Since the total plasma protein is about 2.25 or 2.5 times greater than the plasma protein in circulating plasma, it is tentatively concluded that the skin accounts for about 25 per cent of the total extravascular plasma protein.

Dynamics of the exchange between plasma and skin. The results shown in Fig. 1 and other experiments not described in detail, in which ^{14}C-labelled proteins, labelled antibody and horse serum were used, indicate that a steady state of the relative activity of skin serum proteins as compared with circulating serum is reached about 8 days after injection This steady state probably means that complete equilibration is being approached. The differences between animals are too great to permit a mathematical analysis of the data of the type discussed recently by Campbell, Cuthbertson, Mathews and McFarlane (1956) and we do not know whether the exchange reaction can be described by one first-order reaction. In other words we do not know whether skin is homogenous with respect to exchange of plasma protein. If the reaction were simple and of first order, the 'half-life' of skin serum protein with regard to exchange with plasma, is likely to be about 4 days. These very rough calculations apply mainly to albumin in skin, and no estimates can yet be given for the globulins of skin or for plasma proteins in other types of connective tissue.

SUMMARY

The presence of plasma protein has been demonstrated qualitatively by electrophoresis and serologically in tendon and in skin.

The albumin fraction has been isolated in a partially purified form and shown to have similar properties as the albumin isolated from serum. The amount of plasma protein in rabbit skin is calculated to be about 1 gm. per 100 gm. skin, and to account for about 25 per cent of the total extravascular plasma protein. The rate of exchange between the plasma protein, particularly the albumin between skin and plasma has been measured by using suitably labelled proteins. Results indicate that equilibration may be approached in about 6-8 days.

GROUP DISCUSSION

DR. GRASSMANN said that very small amounts of the component moving faster than albumin on electrophoresis ('pre-albumin') were present also in plasma and that larger amounts could be detected in extra-vascular fluids such as cerebrospinal fluid.

In reply to a question from Dr Grassmann, DR. NEUBERGER stated that the albumin isolated from skin was identical with plasma albumin in its ultraviolet spectrum and tyrosine and tryptophan contents but that a complete amino-acid analysis had not yet been done.

In reply to Dr. Snellman, DR. NEUBERGER stated that he had no evidence that iodination affects the properties of albumin so long as the iodination is only slight and is carried out under the conditions given by MacFarlane in the *Biochemical Journal*.

DR GROSS referred to the work of Gitlin who used the fluorescein-labelled antibody method of Coons which indicated that fibrinogen, some of the globulins and possibly albumin occurs inside the cells, possibly in association with lipids.

He also referred to work of Boas who found approximately 4 per cent plasma proteins in the subcutaneous tissue of the rat, even though there is a negligible quantity of blood.

DR. BOWES asked if the complete removal of activity on extraction with phosphate buffer referred to both the ^{14}C-labelled glycine and the iodinated protein or just to the iodinated protein.

DR NEUBERGER stated that no work had b[illegible] skin after labelled gly[illegible] f synthesized [illegible] After complete ext[illegible], only a very small amount of the iodinat[illegible]mained in the skin residue.

DR D. S. JACKSON stated that in a neutral salt extract of the granuloma produced by injection of carageenin he had found an albumin not quite

identical with plasma albumin and suggested that it was similar to the albumin found in subcutaneous tissue by Dr. Neuberger.

Dr. Orekhovitch said that, for solving the problem of the identity of the blood plasma proteins with some of the skin proteins, a complex method should be applied, depending on a combination of paper electrophoresis or paper chromatography with immunobiological methods. This complex method was worked out by Gurvick, and a partial description of it was published in *Clinica Chimica Acta*.

STUDIES ON THE FIBROGENESIS OF COLLAGEN. SOME PROPERTIES OF NEUTRAL EXTRACTS OF CONNECTIVE TISSUE[1]

JEROME GROSS[2]

Revelation of the mechanism of fibrogenesis has seemed tantalizingly close during the past three decades primarily through morphological studies including both light and electron microscopy, of embryonic growth, wound healing and tissue cultures However, the process and site of polymerization of collagen molecules to fibrils are still undisclosed

One approach to this problem has been made via the study of the behaviour of collagen in solution particularly its reconstitution or precipitation in the form of characteristically striated fibrils as visualized by electron microscopy (Schmitt *et al.*, 1942, Bahr, 1950, Noda and Wyckoff, 1951; Gross *et al.*, 1952, Vanamee and Porter, 1951). Schmitt, Gross and Highberger (1955a, b) have characterized what they believe to be the fundamental building block of the fibril called by them 'tropocollagen' (Gross *et al*, 1954). As deduced from electron microscopic studies on the precipitation and interconversion of several different 'long spacing' and native forms of collagen from solution (Highberger *et al.*, 1951; Schmitt *et al.*, 1953; Gross *et al.*, 1954), this particle is a rigid rod of the order of 2000-3000 Å long and less than 50 Å wide. These observations were made on collagen derived from three different mammalian species and one fish (carp swim bladder tunic from which ichthyocol is derived). Physical chemical studies on ichthyocol by Boedtker and Doty (1955, 1956) confirmed the existence of such a structure in acid citrate solutions at low concentrations. Their measurements on dilute solutions indicate the presence of a remarkably homogeneous population of relatively rigid rod-shaped particles, 2900 Å long by 14 Å wide, with a molecular weight of 340,000 Hall (1956) has

[1] This is publication No 208 of the Robert W Lovett Memorial Laboratories for the Study of Crippling Diseases, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts Grants in support of these investigations have been received from the National Institute of Arthritis and Metabolic Diseases, U S Public Health Service Grants A 90 (C5-6)

[2] Established Investigator of the American Heart Association, Inc , New York.

acteristic collagen fibril.

Earlier physical chemical studies on acid solutions of collagen led to much higher molecular weights, above 1,000,000 (M'Ewen and Pratt, 1953; Gallop, 1955a). There is reason to believe that differences in preparation were responsible for this discrepancy. Gallop (1955b) has shown that gentle warming of an acid solution of ichthyocol will reduce the molecular weight to 70,000 with an abrupt fall in optical rotation and an irreversible loss of ability to reconstitute fibrils.

The term 'tropocollagen' does not imply a precursor role. These particles may be obtained either by dissolving fibrils or by extracting them from the tissues before polymerization; in the latter case they would be precursors of the fibrils.

Because native collagen fibrils are relatively insoluble at neutral pH the discovery of a form of collagen extracted from fresh connective tissue by cold neutral salt solutions (Highberger *et al.*, 1951; Gross, *et al.*, 1955) suggested the presence in the tissue of a form of collagen in the dispersed state, perhaps dissolved in the ground substance. Studies by Harkness *et al.* (1954) on the turnover rate of isotopically labelled collagen in rabbit skin and by Jackson and Slack on carrageenin-induced granulomata in rats, indicated a relatively large and rapid turnover in the neutral salt-extracted collagen, much less in the acid-extracted fraction, and practically none in the insoluble residue. The metabolic activity of the neutral salt soluble fraction indicates the likelihood of its being a precursor of the fibrils. If this hypothesis is correct there should be considerable variation in the amounts of neutral salt-extracted collagen related to growth and certain disorders manifested in the connective tissue such as scurvy.

The aim of this paper is to describe some of the properties of cold neutral salt extracts of fresh connective tissue, and to illustrate the effect of growth rate on the extracted components.

GENERAL METHODS OF PREPARATION AND ANALYSIS

The studies reported here were all performed on albino guinea-pigs ranging in weight from 300 to 500 grammes. The animals were

fed with standard guinea-pig pellets [illegible]
fresh lettuce and 50 mg. of [illegible]
Weight was recorded daily. [illegible]
suspended in 2 volumes of [illegible]
hours, centrifuged at 50,000 g. [illegible] for 15
filtered through coarse, [illegible]
All operations were performed at about 5° C. [illegible]
determination samples were [illegible]
hours. Aliquots of the [illegible] were [illegible] for [illegible]
electrophoresis and ultracentrifugation [illegible]

Viscosity was determined at 5° C. in Ostwald [illegible]
meters with flow times of [illegible] and [illegible]
Electrophoresis in a Perkin-Elmer [illegible] at
3° C. on aliquots dialysed against a [illegible] buffer
$\Gamma/2 = 0.2$, pH 8.6.

Ultracentrifugation was performed [illegible]
centrifuge at 56,100 g. at 5 to 8° C. [illegible] analytical
simultaneously with the aid of a [illegible]

Chemical analyses[1] were performed [illegible]

Hydroxyproline – Neuman and [illegible]
Tyrosine – Bernhardt [illegible]
Hexose – Friedman [illegible]
Hexosamine – Boas (195[illegible]
Morgan [illegible]
Uronic acid – Fishman et al. [illegible]

The electrophoretic pattern of a [illegible] Eston and
0.45, pH 7.6) extract of guinea-pig [illegible]
that of guinea-pig serum [illegible] ($\Gamma/2 =$
slowly diffusing peak in the γ-[illegible] resembled
differences in relative amounts [illegible] and
seen by comparing the ascending [illegible] obvious
that of the extract (Fig. 1b). [illegible] readily
revealed essentially the same [illegible] (Fig. 1a) with
peak (Fig. 1c). Warming [illegible] 0.14M NaCl
duced a rigid opaque structure [illegible] collagen
[illegible] two hours pro-
[illegible] et al., 1955).

[1] All analyses were performed on aliquots [illegible]
low level.

Uronic acid values are only good for [illegible] hyaluronic and [illegible]
oitin sulphuric acids yielded only [illegible] of uronic acid with the
lorimetric method used. The Dische [illegible] methods were [illegible]
plied because of the mixture of unknown [illegible]

This was centrifuged at high speed and the supernatant flı again examined by electrophoresis. Fig. 1*d* shows the maj change to be the near-loss of the high spike in the γ-globul fraction. This solution was no longer viscous and its hydrox proline content had fallen from 131 γ/cc. to 33 γ/cc. The lc

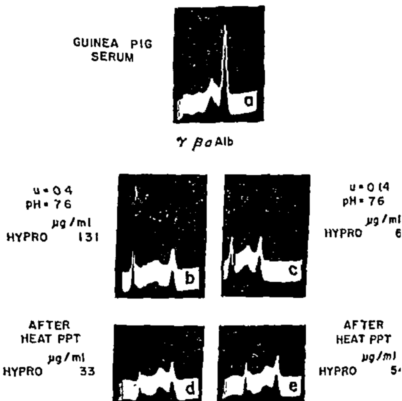


Fig. 1

hydroxyproline was found in the fibrous precipitate. Electron microscopy of this material revealed it to be composed primarily of collagen type fibrils having the usual 640 Å period plus much

amorphous debris. It thus appeared that the slow-moving, high, hypersharp peak was the extracted collagen fraction. Fig. 1e is the pattern obtained from the supernatant after heating the low ionic strength extract (1c) for the same period. Only a small amount of collagen was precipitated, most likely because of the much lower concentration.

Ultracentrifuge patterns obtained before and after heat precipitation also yielded similar results. Here the extracted collagen is manifested as a hypersharp, slowly sedimenting boundary as shown in Fig. 3.

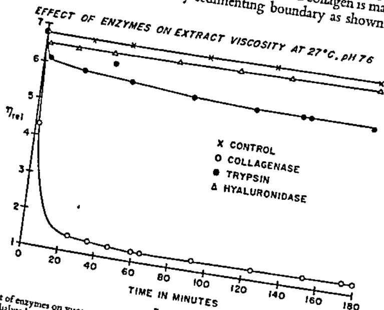


Fig 2

ffect of enzymes on viscosity of 0 45M extract at 27° C as a function of time The extract as dialysed against phosphate Γ/2 = 0 45, pH = 7 6 prior to experiment.

In an effort to characterize these extracts further, the effect of llagenase, trypsin and hyaluronidase on viscosity, sedimentation d electrophoretic pattern was examined. A 0.45M NaCl extract s dialysed vs. phosphate buffer Γ/2 = 0.45, pH 7.6, and split into r equal aliquots. The temperature was raised to 27° C. in a water n and collagenase (1 mg./cc.), trypsin (1 mg./cc.) and hyaluroni- (1 mg./cc.) were added to each of three tubes (dissolved in l volumes of buffer) and the same volume of buffer alone added

to the fourth tube. Viscosity readings were made at frequent intervals in an Ostwald viscometer at 27° C. Results are shown in Fig. 2. Viscosity fell rapidly in the collagenase-treated tube to a minimum within 20 minutes and levelled off at a value close to that of the buffer. There also was a rapid but much smaller fall in viscosity of the trypsin-treated sample to a minimum in 5 minutes followed by a slow, linear decrease. A small, but repeatable, and nearly instan-

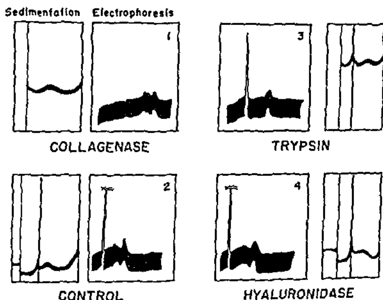


FIG 3
Ascending electrophoretic patterns and ultracentrifuge tracings of enzyme-digested extracts and control

taneous, fall was noted in the viscosity of the hyaluronidase-treated sample as compared with the control. Electrophoretic and sedimentation patterns of all four preparations after enzyme treatment are illustrated in Fig. 3. It is evident that the collagenase attacked the collagen peak primarily, although there appeared to be some modification of the rest of the pattern. Trypsin seems to have reduced the height and increased somewhat the diffusability of the collagen peak. There is also obvious although incomplete destruc-

tion of the non-collagenous components Hyaluronidase seems to have no obvious effect on either pattern.

Attempts to heat-precipitate these preparations at 37° C. produced neither precipitate nor gel in the collagenase-treated sample, a heavy, fibrous, non-gelled precipitate in the trypsin-treated preparation,

collagen although Gustavson has demonstrated a lowering of the shrinkage temperature of fibrous collagen as a result of such treatment. The effect in these experiments was manifested by a rapid but limited fall in viscosity, small changes in the electrophoretic appearance of the collagen boundary and also by the absence of gelation on warming to 37° C. The fibrous precipitate which did form, contained both non-striated and striated fibrils; however, more careful electron microscope study is in order. This experiment

cent hexose and 0.2 per cent hexosamine. The effect of the enzymes on viscosity and heat gelation were essentially the same as that on the crude tissue extract. The question as to whether or not trypsin attacks the collagen molecule directly in a specifically restricted manner or through an intimately associated non-collagenous substance, awaits repetition of this experiment on a more pure collagen preparation.

An important question raised is the source of the collagen extracted.

ground guinea-pig corium were extracted with cold NaCl, NaSCN and Na_2SO_4, all $\Gamma/2 = 0.45$. The most effective medium was Na_2SO_4, the other two were somewhat less effective, but about equal, as manifested by viscosity and hydroxyproline content of the extracts. This experiment strengthened the view expressed earlier that neutral salt-extracted collagen does not derive from the ordinary fibrils. It is worth noting here that saturated Na_2HPO_4 used by Harkness *et al.* (1954), is a much less effective extractant than any of the three salts mentioned above. Borate at ionic strength

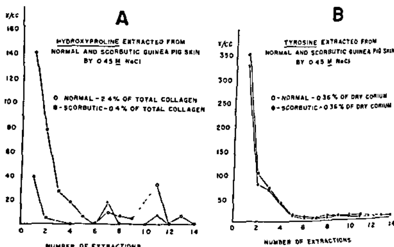


FIG 4
Non-dialysable hydroxyproline (collagen), A, and tyrosine (non-collagenous protein), B, extracted from a scorbutic guinea-pig corium as compared to that from the skin of a pair-fed control Extracting medium was 0 45*M* NaCl

0.45, pH 7.6 extracts negligible amounts of collagen under these conditions.

It was reasoned that if the neutral salt-extracted collagen was truly a precursor of the fibrils, this fraction should be involved in the scorbutic process. If scurvy is characterized by an inability to synthesize collagen there should be either a deficiency or an alteration in properties of the precursor. A group of young guinea-pigs was placed on a vitamin C-free diet and another group of controls was pair-fed with them. A daily weight record of all animals was kept. After 23 days the scorbutic animals were nearly moribund. Histologic assay of the mandibles revealed total scurvy in the test animals, and

one in the controls The skins of the scorbutic and control animals were processed as described, using cold 0 45M NaCl as an extractant The tissues were extracted repeatedly until hydroxyproline and tyrosine were no longer dete[...] the substantial difference in [...] scorbutic animal and its pair-f[...]

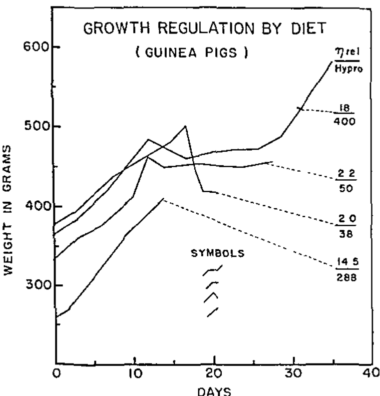


Fig 5
Representative protocols of growth curves of guinea-pigs used in series of experiments on the effect of growth rate on neutral salt-extracted collagen

in the amount of extracted tyrosine, a measure of non-collagenous protein. However, a similar study carried out on rat skin, kindly furnished by Dr Henry Goldman from animals used for other purposes in a nearly identical dietary experiment, revealed a similar, although somewhat less marked difference in extractible collagen

between growing and non-growing rats. It was evident that growth rate was an important factor in the scurvy experiments and review of the protocols revealed that the pair-fed controls were still growing very slowly during their last five days, while the scorbutic animals were losing weight rapidly. However, physical chemical analyses showed changes which could be distinguished from those related to growth. These are now under study.

The following experiments were devised to evaluate the effect of

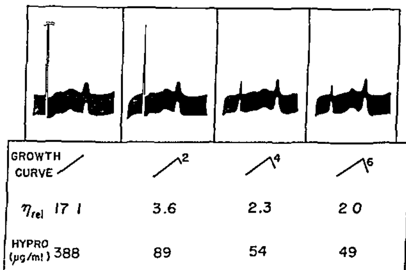


Fig 6

Electrophoretic patterns (ascending limb), viscosity and hypro content of extracts from the skins of guinea-pigs starved for varying periods of time after a long period of active growth Symbols indicate characteristics of weight curve Numbers on symbols indicate the number of days of weight loss

growth on connective tissue components, particularly collagen extracted with cold neutral salt solutions.

Guinea-pigs of about 300 grammes in weight were allowed to grow rapidly for a period of 10 days on an *ad lib.* diet (Group A). They were sacrificed during the period of rapid growth. Another group (B) had growth interrupted by a sharp diminution of intake so that they remained practically static in weight for 8 days, then sacrificed. A third group (C) prepared in a similar manner to that of Group B was then allowed to grow rapidly again for 8 days after the period of static weight before sacrifice. A fourth group (D) was

usual extracts were prepared, viscometrically and analysed and hexosamine and uronic acid. One representative extract from each group was studied in the ultracentrifuge and electrophoresis apparatus.

NaCl EXTRACTS
OF
GUINEA PIG CORIUM

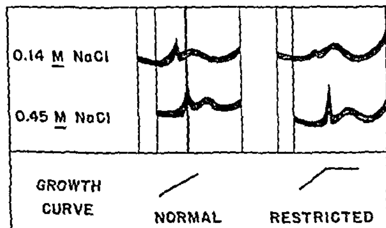


FIG 7
Ultracentrifuge tracing of pattern obtained from extract of skin of rapidly growing guinea-pig as compared to that of a non-growing animal.

The results within each group were quite consistent. Fig 5 illustrates one representative growth curve from each group and the viscosity and hydroxyproline contents of the extract from the skin of that particular animal. Fig. 6 allows comparison of electrophoretic patterns, viscosity and hydroxyproline data from extracts of representative animals of Groups A, D, E and F to show the effect of weight loss. Starvation for two days produced a marked drop in

viscosity and hydroxyproline. This is manifested in the electrophoretic pattern by a fall in the height of the collagen peak; the rest of the pattern is relatively unaltered. Four days of rapid weight loss results in further decrease in these same parameters with little further change in six days. Fig. 7 compares the sedimentation patterns of the extracts of a normally growing animal of Group A and a non-growing animal of Group B. Both low and high ionic strength

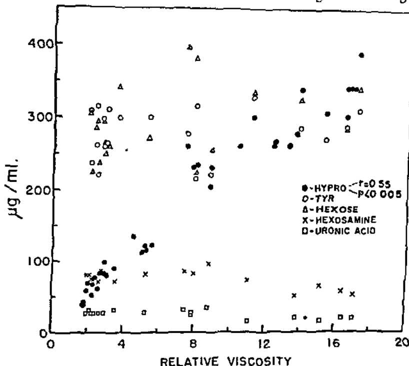


Fig 8

Scatter diagram plotting amounts of extracted, non-dialysable hydroxyproline, tyrosine, hexose, hexosamine and uronic acid as a function of viscosity It is evident by simple inspection that only the hydroxyproline correlates directly with viscosity

extracts are compared. The sharp, slow-moving boundary representing the collagen fraction is markedly diminished in both types of extracts in the static weight animal Viscosity and hydroxyproline content are commensurately low. Animals in Group C, having been allowed to resume growth for 8 days after an equal period of static weight yielded skin extracts of high viscosity and collagen content.

[illegible]

contributing to the viscosity of the extracts Both questions are [illegible]

[illegible]

stituents of collagen. Non-dialysable tyrosine, hexose, hexosamine and uronic acid are not significantly influenced. It should be emphasized, however, that these analyses only measure the quantity of substances present; they reveal nothing concerning properties such [illegible]

DISCUSSION

The case for the precursor nature of neutral salt-extracted collagen is further strengthened by the data presented here, namely that the amount of extractible collagen correlates closely and directly with growth rate, that only two days of starvation with concomitant weight loss results in a many-fold diminution in the amount of salt-extracted collagen. 'Precursor' is here defined as the newly synthesized collagen molecules or small aggregates which have not yet been polymerized in the form of the native 640 Å banded fibrils but are [illegible]

(1[illegible]

alkaline and neutral salt-extracted collagen is strongly in favour of this possibility. That this fraction is readily polymerized *in vitro* into typical collagen fibrils indicates that it is a 'normal' collagen and probably not involved in a separate metabolic cycle for eliminating a faulty protein.

At this time there is no good reason to believe that this is the only precursor form. Certainly within the cells there must be peptide

chains in process of synthesis. It is hypothesized here that such incomplete molecules are incapable of being polymerized into fibrils and it is probably the *fully* formed and functional molecule which is 'secreted' into the extracellular ground substance where polymerization to fibrils occurs. An important question in this connection, as yet unanswered, is whether or not enzymatic modification of this particle is required before polymerization can occur, i.e. a process similar to that involved in the fibrinogen-fibrin transformation. Work is in progress on this phase of the problem.

Preliminary studies in this laboratory by viscosimetric methods indicate that the collagen particles in cold neutral salt extracts of fresh skin are highly asymmetric with axial ratios of the order of 300:1. It is, however, not inconceivable that partial polymerization or aggregation has already occurred in these extracts.

Whether or not any of the other carbohydrate or protein com-

gen fibrils *in vitro* in the absence of anything identifiable as an acid

detected in such preparations; inadequacy of the analytical and

... cannot be excluded, of course.

...obtained in this

...onstituted into

fibrils. Whether or not these substances are integral parts of the molecule or just strongly adsorbed *in a structurally irrelevant* way so far as fibrilogenesis is concerned, is still a question.

... function of the neutral salt-extracted

...cursor, is suggested by

...s. *If all the extracted*

collagen is present in the ground ... a dispersed state rather than in some kind of fibrillar form, its concentration in the extracellular fluid in actively growing animals would be well above 1 per cent. Such a 'solution' of collagen, at least in the test tube, is nearly a gel; it has the consistency of thick molasses. The physical properties of the ground substance should then be strongly dependent upon the amount of neutral salt extractible collagen. In non-growing animals

then, there should be a marked difference in viscosity and other properties, of ground substance as compared to those of actively growing animals.

It is important, however, to emphasize that the neutral salt extraction does not remove all the extracellular substances, and the properties of such extracts may not necessarily reflect faithfully the properties of the intact tissue.

Conclusive correlation between the properties of tissue extracts and the physiological roles they may play awaits the devising of adequate methods for study of the behaviour of these components in the intact fresh tissue.

GROUP DISCUSSION

In reply to a question by Dr Astbury, Dr. Gross said that the salt extract of scorbutic skin did not precipitate at 37° C The collagen in the [illegible] differs from Doty's acid extracts of [illegible] ation in the former. [illegible] lagen in neutral salt solution, even after prolonged [illegible] igation, indicate the presence of tropocollagen aggregates rather than separate units. Doty took particular care to remove aggregates from his material by prolonged ultracentrifugation, but in the case of the neutral salt solution such centrifugation was not effective.

[illegible] S. Jackson said that he and Dr. Fessler had purified collagen [illegible] and had found [illegible] ods in varying [illegible] l the degree of [illegible] This had been [illegible] is material pre- [illegible] ower tempera- [illegible] ed on material [illegible] old room. This [illegible] tic acid. He [illegible] extracts and [illegible] extract contains an extra material which would [illegible] glycine and hydroxyproline contents and the higher tyrosine, serine, threonine and histidine contents. This might be a contaminant or it might be important in the precipitation of this fraction.

Dr. Fitton Jackson suggested that high tyrosine content might be due to contamination by intracellular material

DR. D. S. JACKSON pointed out that his neutral salt extract had been purified and that any such contaminant would have to be present in such large amounts that it would easily be detected by electrophoresis

DR. FITTON JACKSON said that one would expect less contamination in old material which contained fewer cells.

effect can be reversed by iodide or thiocyanate.

TABLE I

SOLUBILITY OF COLLAGEN AND PROCOLLAGEN IN DIFFERENT SOLVENTS

Solvent	*Per cent of dissolved nitrogen* *Collagen* 1st Extr	2nd Extr	3rd Extr	4th Extr	Total	*Procollagen*
Phenol (80 per cent in water)	18 8	—			18 8	easily soluble
Lithium iodide (50 per cent in water)	7 5	3 0	1 6		12 1	easily soluble
Urea (saturated solution in water)	2 5	2 3	3 2		10 0	completely but not easily soluble
Thiourea (saturated solution in water)	1 1	2 5	5 5	4 2	13 3	completely but not easily soluble
Guanidine carbonate (saturated solution in water)		insoluble				insoluble
Guanidine iodide		insoluble				insoluble

this could be repeated several times

DR. GUSTAVSON said that the setting of gelatin solution was markedly

pared the low hydroxyproline content of neutral salt-soluble collagen

with that of collagen in healing wounds in scorbutic animals and also in the skin collagen of certain teleostei (cod) and said that the lack of hydrogen bonds involving hydroxyproline might account for the solubility and low strength of these structures.

Dr Gross referred to a theory propounded first by Robertson that in collagen of scorbutic animals the proline was not properly hydroxylated

[illegible]

gen bonds.

Dr Gross said that collagen becomes more insoluble when it is stored, even in the cold, for a long time This might be due to thermal agitation causing the collagen molecules to assume a more stable configuration within the fibril He suggested that this might also account in part for the increased insolubility of collagen that occurs on ageing under physiological conditions

Dr Neuberger compared this to the ageing of colloidal suspensions.

Dr Orekhovitch said that there was no new formation of procollagen [illegible] agen [illegible] He [illegible] ss in scorbutic animals was due to the non-production of procollagen He [illegible]

[illegible] acid extract gave, after heating, two distinct peaks in the ultracentrifuge

Dr Gross, however, said that when skin was extracted by the Orekhovitch procedure, the pH of the original buffer was identical with that of the final extract

THE FORMATION AND BREAKDOWN OF CONNECTIVE TISSUE

D. S. JACKSON

all

de

been carried out. The recent report by Robertson and Schwartz (1953) that relatively large amounts of connective tissue are formed

developing connective tissue. A concurrent histological study was also carried out (Williams, 1956).

The methods used in this study have been described in detail elsewhere (Jackson, 1956a, b, c, Slack, 1956a, b).

QUANTITATIVE STUDIES

viz.:

(1) neutral salt extracted, (2) released following papain digestion, (3) present in the residue after papain digestion.

Collagen

All three collagen fractions were found, viz.: neutral salt soluble,

at first and more rapidly between the sixth and ninth days. ... mum concentration was reached at 14 days, after which the concentration fell off. However, since the wet weight decreased from days 7-9 onwards the absolute maximum amount of collagen was present about the ninth day.

At 3 days neutral salt-soluble collagen appeared (Table I). The concentration of this fraction increased up to the fifth day and remained constant for 2-4 days before rising again coincident with the [illegible]

TABLE I

VARIATION WITH TIME, AFTER INJECTION OF CARRAGEENIN, OF THE CONCENTRATION OF THE COLLAGEN FRACTIONS EXPRESSED IN MG /100 GM WET WEIGHT OF TISSUE

Time	Wet weight tissue	Total collagen	Neutral soluble Total	Neutral soluble Water soluble	Acid soluble	Insoluble
Days	gm	mg /100 gm wet weight tissue				
3	19 4	14 9	5 5	4 7	0 2	9 2
5	37 9	74 3	18 8	9 7	11 2	44 3
6	56 7	189 3	15 5	6 0	18 2	155 6
7	70 2	333 2	18 9	9 2	28 9	285 5
9	25 5	857 7	58 9	20 3	86 9	711 9
14	23 5	986 1	103 3	41 2	115 1	767 7
21	8 7	1068 3	159 0	58 5	155 2	754 1
28	4.7	717 0	136 5	55 0	148 6	431 9

Total from 3 guinea-pigs

was present throughout the period of the experiment, the concentration/time curve being parallel to that for the total neutral salt-soluble collagen (Table I). The concentration of citrate-soluble collagen, althou[illegible]
the experiment.
creased steadily
the fifth and ninth day. After 14 days its concentration decreased steadily.
[illegible]

soluble form.

TABLE II

RELATIVE PROPORTIONS OF COLLAGEN FRACTIONS EXPRESSED AS PER CENT TOTAL COLLAGEN

Time	*Neutral soluble*		*Acid soluble*	*Insoluble*
	Total	*Water soluble*		
Days	*per cent total collagen*			
3	38.4	30.7	1.6	60.0
5	25.3	13.0	15.0	59.7
6	8.4	3.3	9.6	82.0
7	4.5	2.2	6.9	88.6
9	6.8	2.3	10.1	83.1
14	10.5	4.2	11.7	77.8
21	14.9	5.4	14.5	70.6
28	19.0	7.7	20.7	60.3

TABLE III

AMOUNTS OF $SO_4^{=}$/100 GM WET WEIGHT TISSUE IN THREE FRACTIONS OF GRANULOMA TISSUE AND TOTAL WET WEIGHT TISSUE OBTAINED FOLLOWING SUBCUTANEOUS INJECTION OF 50 MG CARRAGEENIN

(Figures are means of at least three determinations on pooled tissue from three guinea-pigs at each time interval)

Time after injection of 50 mg carrageenin	*Wet weight tissue extracted*	*Salt fraction 1 0.2 M NaCl pH 7.4*	*Papain fraction 2 10 mg papain/gm tissue*	*Residue sulphate 3 by hydrolysis remainder*	*Totals 1, 2 and 3*
Days	*gm*	*mg/100 gm wet weight tissue*			
3	13	23	11	5	39
5	18.5	15	12	5	32
5.5	33	15	13	6	34
6	38	12	13	6	31
7	33	9	11	5	25
9	8.5	7	6	7	20
14	8.5	4	5	8	17
21	3.5	2	3	7	12
28	2.0	1	4	8	13
Normal guinea-pig skin	—	7	9	3	19

Sulphated Polysaccharides

The total bound sulphate of the developing tissue is already at a maximum 3 days after injection of carrageenin (Table III). Salt extractable polysaccharide accounts for 60 per cent of the total at this time, its concentration falling rapidly thereafter. Sulphated polysaccharide released by papain digestion reaches its maximum concentration about the seventh day and falls steadily thereafter. The residue sulphate varies little throughout and would appear not to be concerned in the developing tissue.

ISOTOPE STUDIES

Collagen

The highest specific activity was found in the neutral salt-soluble collagen. Two hours after injection its activity was already high and reached a maximum between 8 and 12 hours (Fig. 1). The activity had fallen rapidly by 24 hours and more slowly thereafter, substantial activity still remaining after 16 days.

TABLE IV

Specific activities of $^{35}SO_4$ in counts/min /0 1 mg $SO_4^=$ found in three fractions from carrageenin induced granuloma in guinea pigs, $Na_2{}^{35}SO_4$, 1 μc/gm body weight, injected intraperitoneally 5 days after 50 mg carrageenin subcutaneously Radioactivities counted with 29 ± 0 3 mg carrier $BaSO_4$ as infinitely thick discs of 1 sq cm surface area

Time after $Na_2{}^{35}SO_4$	*Salt Fraction 1 0 2 M NaCl, pH 7 4*	*Papain Fraction 2 10 mg papain/1 gm tissue*	*Residue Sulphate Fraction 3 by hydrolysis of remainder*
2 hours	12	14	26
6 ,,	354	23	22
8 ,,	462	44	20
12 ,,	672	128	22
24 ,,	586	327	19
2 days	408	303	13
4 ,,	53	245	8
9 ,,	22	199	12
[illegible] ,,	28	143	7
[illegible] ,,	10	115	6

[illegible]he activity of citrate-soluble collagen was low at 2 hours and [illegible] rapidly to a maximum at 12 hours, considerably below the [illegible]mum activity of neutral salt-soluble collagen. The activity/

time curve for insoluble collagen was a little lower than that for citrate-soluble collagen and reached a maximum after 24 hours. Activity had fallen after 48 hours, and after levelling out continued to fall from the fourth day on.

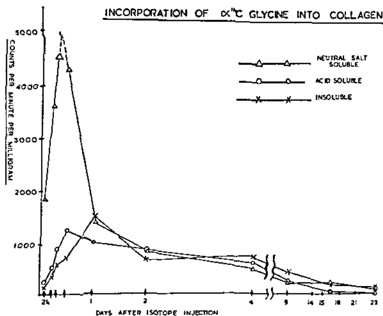


FIG 1

There was some variation in the activities of comparable fractions at comparable times, in experiments carried out at different times, but the activities of the different fractions relative to each other remained the same. Similar findings have been reported in the collagen fractions from rabbits injected intraperitoneally with [α^{14}C]-glycine (Harkness *et al.*, 1954).

Sulphated Polysaccharides

ctivity in about 10 hours. The turnover rate of this fraction is also apid being of the order of 36 hours. The maximum activity of the apain fraction is not reached until about 36 hours, the turnover rate eing about 8 days, similar to that reported for the chondroitin ulphate of rat skin (Boström and Gardell, 1953) and of rabbit skin Schiller *et al.*, 1955, 1956).

DISCUSSION

A comparison of the concentration/time relationship of total collagen (Table I) and total bound sulphate (Table III) suggests that n *the early stages of connective tissue development only small* amounts of collagen (probably of the soluble type) are present and

During the pre-fibrous stage of development the main connective tissue components present are neutral salt-soluble collagen and the sulphated polysaccharides. The salt-extractable polysaccharide may be the precursor of that released by papain digestion, which may be closely associated with the fibrous collagens (Slack, 1956).

The *finding that a proportion of the neutral salt-soluble collagen* was water soluble was unexpected since negligible amounts of hydroxyproline-containing material were found in the same fraction from the skins of young rabbits (Harkness *et al.*, 1954; Jackson, unpublished data). The significance of this finding is not clear but it is possible that either this material represents an earlier stage of collagen synthesis or that it is a lower molecular-weight fraction of neutral salt-soluble collagen which is known to aggregate in solution to varying degrees (Jackson and Fessler, 1955).

Breakdown of Collagen

least from the seventh day. It is possible that both take place simultaneously from the beginning but that synthesis predominates at

first and breakdown later. It has been found that carrageenin injected intradermally causes a rapid breakdown of the dermal collagen fibres without preceding granuloma formation (Williams, 1956). The increase in concentration of both the soluble fractions which occurs concurrently with the decrease in wet weight suggests that the *breakdown of insoluble collagen occurs first by a disaggregation into the units which make up mature collagen fibres*

... ble and meta- ... Slack, 1951). ... hod described here may be inherently unstable. It seems, however, in view of the results reported by Williams (1956), more likely that carrageenin first stimulates the formation of normal insoluble collagen and then stimulates some mechanism which leads to its removal.

Experiments with [α¹⁴C]-Glycine

The results of the [α¹⁴C]-glycine experiments are in complete agreement with the suggestion put forward by Harkness *et al.* (1954) that neutral salt-soluble collagen and not citrate-soluble collagen as suggested by Orekhovitch (1952) is the true precursor of insoluble collagen. The more recent work of Orekhovitch (1955) *reiterating his belief that citrate soluble collagen is the true precursor* ... of extraction used would produce ... separate fractions.

Harkness *et al.* (1954) *also suggested that citrate soluble collagen* and insoluble collagen are not distinct groups of proteins but are both obtained from fibres deposited outside the cell. They also express the opinion that it is unnecessary for all the insoluble collagen to pass through the citrate soluble stage. The activity/time curves obtained in the present study support these ideas, *since those of* citrate soluble collagen and insoluble collagen are very similar and suggest that [α¹⁴C]-glycine is incorporated simultaneously into both fractions.

There are some differences in detail between the present study and that of Harkness *et al.* (1954). The rate of incorporation of [α¹⁴C]-

glycine into the neutral salt-soluble collagen is more rapid, maximum incorporation occurring at about 10 hours as compared with 24 hours for the same fraction from rabbit skin. The activity/time curves for citrate-soluble collagen and insoluble collagen are also different in the two studies, maximum incorporation into acid-

more probably to the higher rate of growth of the carrageenin-stimulated connective tissue.

Harkness *et al.* (1954) believed that the comparatively high activities which they obtained for insoluble collagen at 8 and 24 hours were due to contamination with non-collagen protein of high activity. In this study this difficulty did not arise, the activity of insoluble collagen being low at 2 hours, when the activity of the

collagen. As was shown above (Table I), the decrease in wet weight of the tissue from the seventh day onward is accompanied by a considerable increase in the concentration of soluble collagens. This fact, together with the similarity of the activity/time curves of all three fractions from the seventh to twenty-eighth day suggests that all these components are derived from insoluble collagen during its breakdown. Thus the formation of soluble collagens may be the first stage in the dissolution of collagen fibres.

The Morphological Origin of the Collagen Types

Before discussing the morphological origin, it will be well to review the question of nomenclature and define the terms used to describe the various collagen types. It has become customary to call the fraction extractable with acid buffers 'procollagen', the assump-

fraction will be referred to as 'citrate soluble collagen'.

Greater amounts of collagen can be extracted from skin and tendon with dilute acids than with acid buffers (Bowes *et al.*, 1953, 1956, Jackson, unpublished data). Furthermore, treatment of tendon with

hyaluronidase greatly increases the proportion of this tissue extracted with dilute acetic acid (Jackson, 1953) and only slightly increases the proportion extracted with acid buffers (Jackson, unpublished data).

A further distinction must therefore be made between citrate-soluble collagen and collagen extracted with dilute acid, which will be referred to as 'acid-soluble collagen'. The residue remaining after extraction with neutral and acid buffers and which is usually extracted as gelatin will be called 'insoluble collagen'.

Citrate-soluble Collagen

The morphological origin of insoluble collagen is well known, but the origin of the two soluble types is less clear. Harkness *et al.* (1954) have suggested that citrate-soluble collagen is derived from collagen recently laid down, viz. from the outer layers of large fibres and from the thin argyrophilic fibres which are prevalent in developing connective tissue (Gross, 1950). It has also been suggested by Banga *et al.* (1956) that citrate-soluble collagen is an important constituent of large collagen fibres, playing an important part in the physical properties of the whole collagen fibre. Tustanowski *et al.* (1954, quoted by Banga *et al.*, 1956) believe that citrate-soluble collagen determines the physical properties of the mature fibre which, they suggest consists of a core of 'collastromin' which is structureless, on which are oriented fibrils of citrate-soluble collagen.

Preliminary experiments (Jackson and Williams, unpublished data) involving the histological examination of carrageenin-stimulated connective tissue following extraction with neutral and ... that at least part of the citrate-

buffers, and contains both lipid and carbohydrate (Kramer and Little, 1953; Windrum *et al.*, 1955).

[illegible]

been shown by Schwarz (1956) by electron-optical studies of developing tissue that the silver particles taken up by this type of reticulin are on the surface of the fibre and are topographically related to the D bands of the fibres.

Since microscopically visible fibres are absent when neutral salt-soluble collagen is present (see Table I), this fraction is probably also present in the non-fibrillar part of the connective tissue.

It cannot be decided from the present study whether any of the neutral salt-soluble collagen isolated was originally intracellular. Studies by Porter and Vanamee (1949) using the combined tech- [illegible]

mature form extracellularly by accretion of material from the ground substance. These findings have been confirmed (Fitton [illegible]

stead amorphous masses, having the tinctorial properties of collagen, which are probably antigen-antibody complexes. This would [illegible]

1955) Stearns (1940a, b) concluded on the basis of her observations of fibre formation in a transparent chamber in the rabbit ear, that these granules secreted a soluble precursor into the extracellular space, where this material was transformed into a fibrous form It has recently been shown that neutral salt-soluble collagen can precipitate spontaneously into typical collagen fibres (Jackson and Fessler, 1955). It would seem probable that intracellular collagen is non-fibrous and is secreted into the extracellular spaces from which it can be extracted as a neutral salt-soluble collagen.

Insoluble Collagen

The end product of normal connective tissue formation is the thick collagen fibre which is largely insoluble in most salt solutions. There are probably several factors which determine the solubility

(1953) suggested that chondroitin sulphate might be a stabilizing factor, but later (Jackson, 1954) suggested that a mucoprotein other than chondroitin sulphate might play an even more important stabilizing role This idea has been supported by evidence that a

also suggest that citrate soluble collagen is a stabilizing factor since extraction with acid-citrate buffer reduces the shrinkage temperature by 12° and prevents the complete shrinkage-relaxation cycle characteristic of 'young' fibres, which is absent from 'old' fibres. It is possible, however, that prolonged soaking in acid buffer would itself affect cross-linkages and reduce the shrinkage temperature. It is noteworthy that 'young' human tendon swells considerably at acid pH without any collagen being dissolved (Banfield, 1952).

the role of the ground substance in the process is obscure. The fact that comparatively large amounts of sulphated polysaccharides are present before any visible fibres are formed may indicate that they provide the necessary environment for fibrogenesis.

The fibroblast apparently synthesizes a precursor, possibly the collagen molecule which has been called tropocollagen (*Gross et al.*, 1955). This is secreted by the fibroblast into the extracellular space *from which it is extracted* as neutral salt-soluble collagen. This can be handled in two ways.

(1) Form submicroscopic fibrils which can accumulate further particles of neutral salt-soluble collagen to form visible histological reticulin, i.e. citrate-soluble collagen, in which the cross-linkages are only fairly strong. They may also contain a citrate-soluble form of

protein, now only extractable with alkali (Bowes *et al.*, 1956). The outer fibres most recently laid down may still have only weak cross-links and still be soluble in acid buffers. Finally the whole fibre

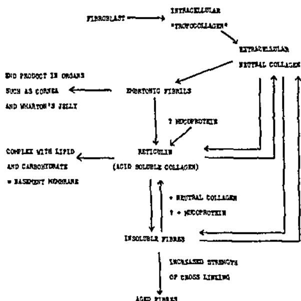


FIG. 2

matures with age, the cross-linkages increasing in strength and

stage citrate-soluble collagen need not be an intermediate between neutral salt-soluble collagen and insoluble collagen.

The first process would probably predominate in the early stages of connective tissue development and the second when the tissue is maturing.

In certain organs the process will stop at earlier stages. In such tissues as the nucleus pulposus, cornea and Wharton's jelly, the *process goes no further than the sub-microscopic fibrils. Similarly* in the reticular tissues the argyrophilic fibres do not develop further, possibly due to the formation of a complex with lipid and carbohydrate.

[illegible]

suitable for investigations into the factors which influence the formation of connective tissue and to discover at which of the stages of *fibrogenesis these factors are effective.*

ACKNOWLEDGMENTS

I am grateful to Professor J. H. Kellgren for his support and to [illegible]

GROUP DISCUSSION

[illegible] the collagen [illegible], reveals the presence of branching argyrophilic fibres which cannot be seen in the compact tissue He suggested that the immature 'reticulin' fibrils are never enlarged to collagen fibrils but are overwhelmed statistically by the [illegible] laid down as the animal [illegible] mucopolysaccha- [illegible] soluble collagen which did not contain polysaccharide nevertheless may show argyro-

[illegible] He thought the argyrophilia was due to the outer part of the

connective tissue

In reply to Dr. Meyer, Dr. Jackson said that as far as extraction pro-

buffer of the same pH results in different material. This is suggested by the very great swelling of the fibres that occurs in the case of acetic acid extraction Acetic acid dissolves a larger fraction of the fibres Later Dr. Bowes said that the protein extracted with citrate buffer, pH 3.7, and acetic acid, pH 2 8, were different. With citrate buffer the protein extracted decreased in successive extracts, whereas with acetic acid there was no indication of such a decrease and it looked more as if the fibres as a whole were very slowly going into solution.

Dr Jackson agreed that the property of the extract depended on the method of extraction

In reply to Dr Schwarz, Dr. Jackson said that the procedure for silvering the sections was based on the method of Lillie. Dr. Schwarz

dicated that the silver was on the surface

Dr. Schwarz referred to electron-microscope work which showed that when interfibrillar cement was removed, for instance, by treatments with hyaluronidase, argyrophilia was also removed.

Dr. Robb-Smith said that injection of methyl cellulose produced a similar tissue effect to carageenin but that the methyl cellulose remains

In certain organs the process will stop at earlier stages. In such tissues as the nucleus pulposus, cornea and Wharton's jelly, the process goes no further than the sub-microscopic fibrils. Similarly in the reticular tissues the argyrophilic fibres do not develop further, possibly due to the formation of a complex with lipid and carbohydrate.

suitable for investigations into the factors which influence the formation of connective tissue and to discover at which of the stages of fibrogenesis these factors are effective.

ACKNOWLEDGMENTS

I am grateful to Professor J. H. Kellgren for his support and to

GROUP DISCUSSION

In reply to a question from Dr. Robb-Smith, Dr. Jackson suggested that neutral salt extraction removed the outer part of the argyrophilic fibres without dissolving the fibres completely and that it is the outer components of the fibres that are responsible for argyrophilia He suggested that the silver is adsorbed on the surface of the fibre.

Dr. Gross said that treatment with trypsin also prevents the argyrophilia and suggested that the argyrophilia was a property of an extracollagenous material

Dr. Gross added that the injection of saline which spreads the collagen fibres apart, as observed by Ranvier and later by Nageotte, reveals the presence of branching argyrophilic fibres which cannot be seen in the compact tissue He suggested that the immature 'reticulin' fibrils are never enlarged to collagen fibrils but are overwhelmed statistically by the

the animal
polysaccha-
ible collagen
ow argyro-

ably. He thought the argyrophilia was due to the outer part of the

ment membrane and the argyrophilic fibres in developing or regenerating connective tissue.

In reply to Dr. Meyer, DR JACKSON said that as far as extraction procedure and analysis for hydroxyproline went, the neutral salt soluble collagen from granuloma is indistinguishable from that extracted from normal skin, but that he regards the material extracted from granuloma during the resorption process as a stage of breakdown of collagen and that from normal skin and from the early granuloma as a stage in the synthesis of collagen.

Alginic acid also produces the same reaction as carageenin but this is the only other high-molecular weight compound known to have this effect. In reply to Dr Glynn, DR. JACKSON said that it was not yet known

acetic acid, pH 2 8, were different. With citrate buffer the protein extracted decreased in successive extracts, whereas with acetic acid there was no indication of such a decrease and it looked more as if the fibres as a whole were very slowly going into solution.

DR. JACKSON agreed that the property of the extract depended on the method of extraction

In reply to Dr Schwarz, DR JACKSON said that the procedure for silvering the sections was based on the method of Lillie. Dr Schwarz said that from the light microscope work alone it is impossible to say whether silver was deposited on the surface or was actually inside the fibrils Dr Jackson agreed but said that the fact that the ability to take up silver is lost without apparently altering the structure of the fibre indicated that the silver was on the surface

DR. SCHWARZ referred to electron microscope work

DR ROBB-SMITH said that injection of methyl cellulose produced a similar tissue effect to carageenin but that the methyl cellulose remains

In certain organs the process will stop at earlier stages. In such tissues as the nucleus pulposus, cornea and Wharton's jelly, the process goes no further than the sub-microscopic fibrils. Similarly in the reticular tissues the argyrophilic fibres do not develop further, possibly due to the formation of a complex with lipid and carbohydrate.

It would also appear from the work described above (see p. 67)

suitable for investigations into the factors which influence the formation of connective tissue and to discover at which of the stages of fibrogenesis these factors are effective.

ACKNOWLEDGMENTS

I am grateful to Professor J. H. Kellgren for his support and to Dr. H. G. B. Slack who carried out the work on the sulphated polysaccharides and kindly allowed me to use his data before publication and to Dr. G. Williams who did the histological study.

GROUP DISCUSSION

In reply to a question from Dr. Robb-Smith, DR. JACKSON suggested that *neutral salt extraction removed the outer part of the argyrophilic* ... it is the outer ... yrophilia He ... ibre

DR. GROSS said that treatment with trypsin ... its *the argyro-philic* and suggested that the argyrophilia was a property of an extra-

... ich spreads the collagen ... y Nageotte, reveals the ... cannot be seen in the compact tissue. He suggested that the immature 'reticulin' fibrils are never enlarged to collagen fibrils but are overwhelmed statistically by the ... fibrils laid down as the animal ... *polysaccha-*

... ible collagen ... ow argyro-

STRUCTURAL PROBLEMS ASSOCIATED WITH THE FORMATION OF COLLAGEN FIBRILS *IN VIVO*

Sylvia Fitton Jackson

An investigation of the elaboration of the fibrils of collagenous tissues involves three main issues; firstly, whether the collagen molecules and their precursors are synthesized solely within the cell and, if so, what specific organelles are concerned; secondly, how the collagen fibrils are formed from these precursors and whether the transformation is intra- or extracellular, thirdly, by what mechanism the collagen fibrils continue to enlarge, and form into bundles. The results described in this paper are concerned with these questions

Studies on both living and fixed tendon and bone tissue of avian embryos have demonstrated the presence of many cytoplasmic granules in the collagen-forming cells, when intercellular material is about to be or is being deposited Qualitative cytochemical methods [illegible]

[illegible]

strongly basophilic in most of their cytoplasm; this indicates the presence of ribonucleoprotein

The differentiation of collagen fibrils is a morphological problem, and it has been followed by studying thin sections of developing avian tendon in the electron microscope. These observations on fibrogenesis in tendon tissue (Fitton Jackson, 1956a) show that filaments, of 80 Å in diameter, which are the first to be distinguished in 8-day embryos, differentiate into characteristic collagen fibrils. The development of the fibrous collagen seems to be closely correlated with the amount of extracellular space that is available; thus the first filaments are either within or intimately associated with the cytoplasm since there are no clearly defined intercellular regions. *As age increases, however, intercellular areas appear (Fig. 1); they* presumably contain interstitial fluid and they gradually become filled

and becomes encapsulated whereas the carageenin is absorbed and the tissue reaction is resolved.

Dr Orekhovitch *said that Dr. Jackson's data concerning the content* in connective tissue of neutral salt solution-soluble, acid-soluble and insoluble collagens, after carageenin injection, does not justify the conclusion that the precursor of insoluble collagen is neutral salt-soluble collagen, rather than procollagen. The low content of procollagen during the

conversion of neutral salt-soluble collagen through acid-soluble collagen

geenin consists of two fractions, one a polygalactose and the other a

in which 10 per cent of the galactose was replaced by the

with Dr. Astbury that they were not similar to polysaccharides which had been isolated from connective tissue Dr. Gross suggested that it might be possible to distinguish between neutral salt-extracted collagen formed

water-soluble collagen but Dr. Jackson said that the

his water-soluble collagen could not be removed by prolonged dialysis

Dr. Baló referred to mucous granulomata in the lungs. These differ from those caused by carageenin in that they do not disappear after a time.

STRUCTURAL PROBLEMS ASSOCIATED WITH THE FORMATION OF COLLAGEN FIBRILS *IN VIVO*

SYLVIA FITTON JACKSON

An investigation of the elaboration of the fibrils of collagenou tissues involves three main issues; firstly, whether the collagen molecules and their precursors are synthesized solely within the cell and, if so, what specific organelles are concerned; secondly, how the collagen fibrils are formed from these precursors and whether the transformation is intra- or extracellular; thirdly, by what mechanism the collagen fibrils continue to enlarge, and form into bundles. The results described in this paper are concerned with these questions.

Studies on both living and fixed tendon and bone tissue of avian embryos have demonstrated the presence of many cytoplasmic granules in the collagen-forming cells, when intercellular material is about to be or is being deposited. Qualitative cytochemical methods have shown that the granules contain both protein and mucopolysaccharide, they appear to be capable of chemical synthesis for they also contain alkaline phosphatase and cytochrome oxidase. It has been postulated that the granules have fibrogenic properties and are concerned in the synthetic processes associated with the formation of the intercellular materials (Fitton Jackson, 1955). The cells are also strongly basophilic in most of their cytoplasm; this indicates the presence of ribonucleoprotein

The differentiation of collagen fibrils is a morphological problem, and it has been followed by studying thin sections of developing avian tendon in the electron microscope. These observations on fibrogenesis in tendon tissue (Fitton Jackson, 1956a) show that filaments, of 80 Å in diameter, which are the first to be distinguished in 8-day embryos, differentiate into characteristic collagen fibrils. The development of the fibrous collagen seems to be closely correlated with the amount of extracellular space that is available; thus the first filaments are either within or intimately associated with the cytoplasm since there are no clearly defined intercellular regions. As age increases, however, intercellular areas appear (Fig. 1); they presumably contain interstitial fluid and they gradually become filled

with fibrils until finally bundles are formed. In longitudinal sections of 11-day embryonic tendon the fibril diameter is about 120 Å and the axial periodicity of immature collagen, viz. about 210 Å, is discernible, and agrees with that found in *teased preparations taken* [illegible] gh angle X-ray diffrac- [illegible] these embryos gives a recognizable fibre diagram for collagen (Fig. 2); the characteristic spacing of 2.86 Å is apparent and with increasing age the equatorial spot at 12 Å becomes more clearly defined.

Each collagen fibril within a bundle is surrounded by a structureless substance of low electron *density; this has been termed inter*fibrillar material. Within the cross-section of the adult fibril a number of smaller units are sometimes visible, but their size and [illegible] clearly defined (Fig. 3). Studies by [illegible]

chains) into regularly arranged bundles. Thus it is tentatively suggested that the structural level of the units observed in the cross-section of adult fibrils may be intermediate between that of the [illegible]

[illegible] increases with age (Fig. 4) and that such enlargement is accompanied by a reduction in the relative amount of interfibrillar material which invests each fibril. The ratio of these substances may be defined briefly as the packing fraction of the fibrils within the bundles since it gives a measure of *the degree of close packing of the fibrils and it* has been plotted as a function of age (Fig. 4).

By the use of sections in which fibrils have been cut exactly transversely to the bundle axis it has been possible to investigate more accurately the relationship of these fibrils to the cells during the process of fibrogenesis. The morphological picture indicates that fibrils are formed both intra- and extracellularly. Firstly, groups of *fibrils have been found well* within the cytoplasmic areas (Fig. 5); these are ultimately extruded from the cell by a method that is not *known. Secondly, fibrils arise* outside the cell but often in close association with the cell surface; this method of elaboration predominates as age increases. During differentiation (Fig. 6) the cytoplasm *of adjacent cells and the bundles of* collagen fibrils are so

intimately related to each other that a jigsaw outline is obtained. If the cell surfaces are traced it becomes evident that the bundles are inter-connected by narrow channels of not more than 400 Å across so that the extracellular regions are continuous with each other. During development, it is noticeable that there are only small variations in the diameter of the individual fibrils at any one age

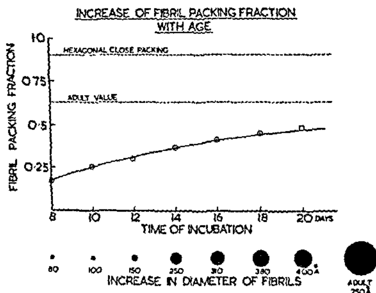


Fig 4

Diagram of (a) the rate of increase in the diameter of collagen fibril with age and (b) the curve of the packing fraction of the fibrils in a bundle with age. The packing fraction of long solid cylinders is marked for comparison purposes as well as the figure for adult tendon (0·62)

which suggests that the fibrils must have been formed almost simultaneously or by a very strictly controlled mechanism.

As collagen-fibre bundles, detectable by light optical methods appear, a positive periodic acid-Schiff reaction is at first obtained from the intercellular regions, these areas also stain metachromatically, and the intensity of the stain increases as the regions enlarge. The intense metachromasia is then gradually lost and in the adult fowl it can only just be detected.

The formation of organized tissue involves various stages of differentiation. It is suggested that the first stage is one of chemical synthesis of the macromolecules, the second stage may be resolved by electron microscopy, and consists of the organization of the collagen and other molecules into fibrils and interfibrillar material; the final stage may be observed by light microscopy and includes the differentiation of the cells and intercellular material into the distinctive tissue.

In the present study, some evidence has been provided of probable synthesis within the fibrogenic cells of collagen protein or its precursor and of mucopolysaccharide and it is apparent that the cytoplasmic granules may be concerned in these synthetic actions. Thus it is believed that the first stage of differentiation may be represented by the formation of precursors and/or collagen molecules as a secretory product of the cells.

The method of the organization of the intercellular material as seen in thin sections of tissues suggests that it is formed when the cellular secretions come into contact with interstitial fluid and the transformation of the molecules into their fibrous form may thus be due solely to a change in environment, the interstitial fluid supplying, e.g. a medium of appropriate ionic strength and pH. This conversion may occur as a direct result of either the extrusion of secretions into the intercellular regions and subsequent reaction with the fluid, or, since Lewis (1941) has shown that the surrounding fluid may be introduced into the cell by pinocytosis, the cell secretions may come into contact with such fluid within the cytoplasm, and, after transformation would appear as an intracellular formation [illegible] formed collagen

to change in environment, but that the interstitial fluid may provide additional organic and/or enzymatic substances essential for the final stages in the formation of the intercellular matter. The above hypothesis does not necessarily preclude other processes which may also be carried on simultaneously (e.g. Fitton Jackson and Randall, 1956)

The fact that the diameter and packing fraction of the fibrils have been shown to change with age has lead to the conclusion that the interfibrillar material must contain collagen molecules which, as

[illegible]

skin is dispersed in the 'ground substance' of the tissue and is a [illegible]

fibrils is not clearly defined. In sections of early osteogenic tissue,

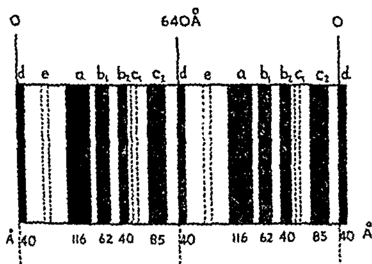


Fig. 7
Diagrammatic representation of the organization of the bands and interbands within two adjacent periods, and the corresponding width of the bands in collagen fibrils of periosteal bone

however, the fibrils clearly show a mean periodicity of about 630 Å with a considerable degree of fine structure (Fitton Jackson, 1956b). The average width of the bands within a period (normalized to 640 Å) has been measured by means of densitometer traces of electron micrographs of newly formed fibrils, and is shown diagrammatically in Fig. 7. The diameter of the fibrils adjacent to the cells in periosteal bone is often about 400 Å, and transverse sections confirm that each fibril is invested by less dense interfibrillar material.

Fibrogenesis in bone-forming tissue is essentially similar to that in

the appropriate part of the fibrils, the particles are seen to be irregular in outline, are about 100 Å apart and number up to 10 per ring. Powder diffraction patterns have been obtained by electron diffraction from such sections, and measurements of the Bragg spacings are in close agreement with those obtained from a control specimen of hydroxyapatite. The particles must be related therefore to the deposition of the apatite content of bone. There is no clear evidence, however, from either electron microscopy or electron diffraction, of any preferred orientation of the particles with respect to the fibre axis, as has been demonstrated by means of X-ray diffraction by

special morphological or chemical characteristic which may account for their ability to calcify.

These studies, however, have failed to demonstrate any notable

sti-

to

gen

but the char-

, was apparently

nterest to try to

determine if there were any features in the methods of fibrogenesis of banded, as opposed to unbanded collagen fibrils, which might account for the observed differences in the morphological structure

periphery of the worm show that the main body of the cuticle is composed of about 18 layers of unbanded fibrils about 1500 Å wide

and orientated in a criss-cross fashion at angles ranging from
106° to those of the adjacent layers. Transverse sections of the
indicate that they are irregular in outline. The fibrils of the c
weave in and out of precisely arranged cytoplasmic processes v
penetrate the cuticle from the underlying epidermal cells and w
stretch between the cells and the outer membrane of the worm.
observations which have so far been made, suggest that the c
plasmic processes may take part in the orientation of the unban
fibrils for it is reasonable to assume that the epidermal cells sec
the precursors of the cuticle.

It has been found by cytochemical methods, that small granu
are present in the cytoplasm of these underlying epidermal cel
These bodies are not the large mucinous globules, which are believe
to be concerned in the production of the mucin which covers th
exterior surface of the worm. The granules are periodic acid
Schiff positive, and may therefore contain polysaccharide. It is pos-
sible that they are homologous with the cytoplasmic granules in
vertebrate collagen-forming cells It is interesting that in their recent
analysis of the earthworm cuticle (*Lumbricus* sp) Watson and Smith
(1956) have shown that its content of hydroxyproline is extra-
ordinarily high, some 50 per cent more than the amount normally
present in mammalian collagen, but it contains very little proline.

At the beginning of this paper it was stated that the problem of fibrogenesis involved three main questions and these questions have in part been answered Firstly, some evidence has been provided which supports the view that the collagen protein molecules and their precursors are formed within the fibrogenic cell and that the cytoplasmic granules are concerned in the synthetic processes associated with the formation of the intercellular material. Secondly, it has been demonstrated that collagen fibrils arise both intra- and extracellularly but mainly the latter. Thirdly, it has been concluded that the interfibrillar material of collagen bundles in vertebrate tissues contains collagen molecules or their precursors, and that these are subsequently deposited on to the growing fibrils in the form and packing appropriate to the characteristic fibre diagram of the collagen protein

GROUP DISCUSSION

Dr. Grassmann asked whether the distances of the various collagen sub-bands correlated with those he had described Dr. Fitton Jackson

replied that in previous work there was, for instance, agreement in their measurements on rat-tail tendon but these measurements differed from those made on fowl-neck and kangaroo tendon. The osteogenic fibrils again show a difference in the width of the bands to those previously reported. Unstained, freshly teased fowl-neck tendon fibrils contained two E-bands whereas osteogenic fibrils contained only one E-band. There might thus be no precise correlation between bands of different tissues and species.

DR. GROSS noted the remarkably constant increase of collagen fibril diameter with age Had Dr Fitton Jackson found any bundles of collagen in older or adult preparations consisting entirely of small fibrils? DR. FITTON JACKSON said, no, she had not. She confirmed the extraordinary regularity in fibril growth. Hundreds of fibrils had been measured for each specimen in each age group, and the width did not vary beyond ten per cent either way In fact, the age of the tissue could be gauge from measurements of fibril diameter in a given section of metatars tendon.

DR. ROBB-SMITH inquired whether the bundles of fibrils which a peared to be intracellular were not really extracellular being enclosed cytoplasm of irregularly shaped cells, in other words 'invaginatio DR FITTON JACKSON replied that although this might occur occasiona with single bundles the occurrence of groups of several, closely pla bundles made it unlikely.

DR GILLMAN inquired as to the nature of the 'clear' areas surround sectioned fibrils in Dr Fitton Jackson's lantern slides.

DR FITTON JACKSON said the evidence indicated that the interfibr material contained collagen molecules or their precursors; it might contain a small amount of polysaccharide, though no data was avail at this point. The methacrylate had not been removed from the sect and therefore the original 68 per cent of water contained in the t should have been replaced by the methacrylate. The clear areas n have been increased by fibril shrinkage as compared with that existi the material *in vivo* but shrinkage must have been of the same o degree as otherwise she would not have obtained such a smooth from the ratio of the diameter of the fibrils to the interfibrillar ma

DR HALL said he was interested in the first stage of differen described by Dr. Fitton Jackson and which she felt might be of a ch nature He pointed out that if one accepted the concept of the proc of a primitive collagen, by the interaction of dissimilar helices, it be at this stage that such a differentiation would occur. His ow gave evidence that this was so In connective tissue brittle, anis fibres with a high content of a polysaccharide indistinguishab cellulose, and containing a protein fraction which on analysis p

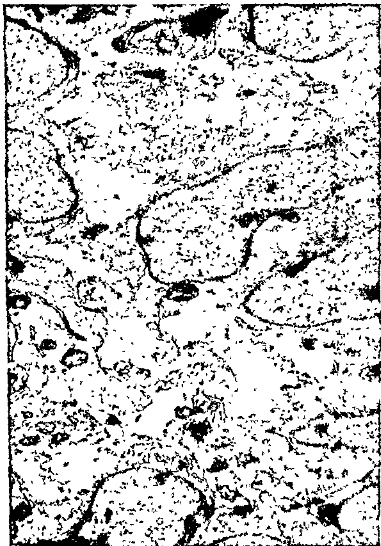


FIG. 1
Electron micrograph of a section of 10-day embryonic tendon: intercellular spaces have begun to appear between the fibroblasts and contain some filaments. × 28,000

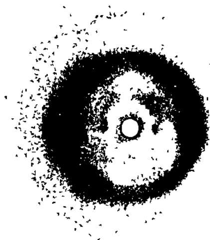


FIG 2
High angle X-ray diffraction pattern obtained from the tendon of an 11-day embryo

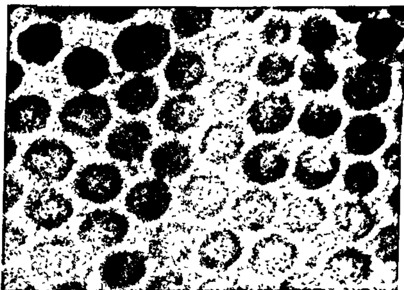


FIG 3
Transverse section of tendon from an adult fowl Each collagen fibril has a distinctive outer boundary and is invested by interfibrillar material × 150,000

FIG 5

In a 12-day embryo, small groups of fibrils are included within the cytoplasm of the cell and there is no evidence of adjacent cell surfaces surrounding the individual fibril groups At the left of the micrograph, the cell surface is apparent × 75 000

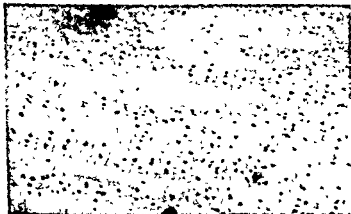


FIG 8

Section of periosteal bone The well-formed structure of the collagen fibril is evident and dense particles are localized in one interband of each period



Fig 6

In an 18-day embryo, bundles of collagen fibrils are lying adjacent and in between two cells, *the respective nuclei are very close to the bundles for there is not more than 200 Å between* them, i e there is only a very thin sleeve of cytoplasm covering the nuclei. × 36,000

[illegible] between collagen and elastin could be demonstrated. The [illegible]

conditions, certain specific polysaccharides intervened at this stage of chemical differentiation to bring about the formation of collagen or elastin fibres from the precursors elaborated by the fibroblasts, it was possible for the wrong polysaccharide to react and become incorporated in the fibre with the production of a 'vitrified' structure

DR. GUSTAVSON commented on the high hydroxyproline content (13 per cent) and the low proline content (1 5 per cent) of the earthworm cuticle, in spite of the 40° C. shrinkage temperature and temperature of dissolution reported by Rudall and Reed. Stabilizing hydrogen bonds involving hydroxyproline might be formed only when the molecules are oriented in a particular manner. The wide hydroxyproline-proline difference is unique.

DR. GROSS stated that the hydroxyproline-proline content of earthworm cuticle described by Dr. Jackson was confirmed independently by Highberger and by himself.

THE ACID MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE[1]

KARL MEYER, PHILIP HOFFMAN AND ALFRED LINKER

An interdisciplinary conference such as this one presents a uniqu opportunity to learn from other specialists problems and viewpoint which are different from those obtained in one's own narrow fielc of experience. In fact, it will undoubtedly take the combined anc

[illegible]

nective tissues and which show promise at growing more numerous in the coming years.

The following table lists the mucopolysaccharides which have

TABLE I

ACID MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE

I	Nonsulphated mucopolysaccharides
	1 Hyaluronic acid
	2 Chondroitin
II	Sulphated mucopolysaccharides
	3 Chondroitin sulphate A
	4 Chondroitin sulphate B
	5 Chondroitin sulphate C
	6 Heparitin sulphate
	7 Keratosulphate

been identified with their characteristic properties and composition. (In this discussion only acidic mucopolysaccharides will be mentioned. Neutral or acid mucoids and glycoproteins which are present in connective tissues will not be considered since no defined compounds have been isolated nor have they been shown thus far to be distinct from plasma proteins) The first group is sulphate-free and includes hyaluronic acid and chondroitin. There is some

[1] From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York, N Y

This work was supported by grants from the National Institutes of Health, the Helen Hay Whitney Foundation and the Eli Lilly Company

evidence for an analogue of heparin which is either sulphate-free or undersulphated. The structure of hyaluronic acid has been fairly well established as an unbranched polymer of a disaccharide repeating unit, N-acetylhyalobiuronic acid, joined by β-4-o-glucosaminidic bonds. The structure of the disaccharide unit has been established as 3-o-(β-D-glucopyranosyluronic acid) 2-acetamido-2-deoxy-D-glucopyranose (Weissmann and Meyer, 1954). Hyaluronic acid, as isolated from different sources such as umbilical cord, synovial fluid, vitreous humor and some mesodermal tumours, does not

microbial hyaluronidase (Linker *et al.*, 1956). No evidence has yet been found for the occurrence in tissue of a *sulphated* hyaluronic acid or of hybrid saccharides of the type produced *in vitro* by the transglycosylative action of testicular hyaluronidase on mixtures of hyaluronic acid and chondroitin sulphates (Hoffman *et al*, 1956). Hyaluronic acid probably occurs in most connective tissues although in greatly varying concentrations. It appears to be the product of the least differentiated fibroblast In hyaline cartilage and in cornea, hyaluronic acid is probably absent.

chondrosine obtained from chondroitin sulphate A. (Its structure will be discussed below.) Chondroitin has only been isolated from cornea.

THE CHONDROITIN SULPHATES

On the basis of solubility, optical rotation and enzymatic hydrolysis, three chondroitin sulphates are distinguished which have been designated as A, B and C.

A and C appear to be closely related. On acid hydrolysis, they

both yield the identical crystalline disaccharide chondrosine, the

Chondrosine has been shown to be the galactosamine containing isomer of hyalobiuronic acid on the basis of the following scheme of reactions in Fig. 1. The first reaction shows that chondrosamine

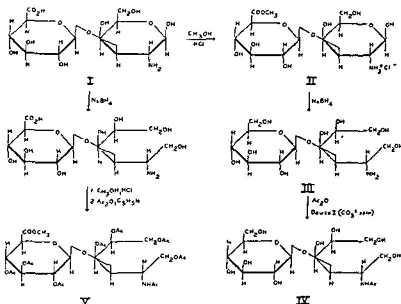


Fig. 1
Structure of chondrosine.

is on the reducing end. Chondrosinol obtained by borohydride reduction of the reducing end group was esterified and the resulting methyl ester was reduced to the alcohol. The resulting glucosido-

chondrosine is D-glucuronic acid. A 1-3 glucuronidic bond in chondrosine was demonstrated by periodate oxidation of a glucosyllyxitol obtained by deamination and subsequent reduction of

chondrosine methylester. The periodate consumption, resulting in the formation of 2 moles of formic acid and of one mole of formaldehyde, is only compatible with a 1-2 link of the glucosidolyxitol. Thus chondrosine was established as 3-O-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-galactose. The galactosaminidic bond is in all probability as in hyaluronic acid a β-1-4 linkage as evidenced from the formation of a 4-5 unsaturated disaccharide on hydrolysis with bacterial hyaluronidase of partly desulphated oligosaccharides obtained on partial acid hydrolysis of chondroitin sulphate (Davidson and Meyer, 1954, 1955). The sulphate group in both chondroitin sulphate A and C is in the galactosamine moiety. The tetrasaccharide obtained as the main product in exhaustive hydrolysis of the chondroitin sulphates (A and C) with testicular hyaluronidase, was digested with β-glucuronidase which cleaved the glucuronic acid moiety from the non-reducing end (Linker *et al.*, 1955). The resulting trisaccharide retained two sulphate groups. Whether the hydroxyl of carbon 6 or 4 is sulphated is not known. The most probable structure of chondroitin sulphate is presented in Fig. 2. The basis for the difference in the properties of A and C is not known. From some tissues chondroitin sulphate fractions were obtained with marked sulphate deficiencies. They were especially abundant in growing (calf) bone. The sulphate deficiency does not appear to be due to loss of sulphate during the preparation. The sulphate deficient fractions are hydrolysed by bacterial hyaluronidases with the production of unsaturated uronides. As in model experiments the enzymes attack the hexosaminidic groups only where the sulphate is missing. Whether the incompletely sulphated fractions belong to the A or C series or to both cannot be decided at present.

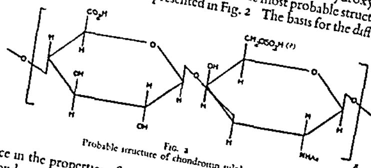


FIG. 2
Probable structure of chondroitin sulphate A

Chondroitin sulphate A has been demonstrated in cartilage, bone,

cornea, aorta and in ligamentum nuchae and in chondrosarcoma. C has been isolated from cartilage, umbilical cord and other primitive connective tissues, tendon, nucleus pulposus (Orr, 1954), a human chordoma and an osteosarcoma.

Chondroitin sulphate B, like A and C, is composed of equimolar concentrations of N-acetyl D-galactosamine, uronic acid and sulphate. It has an optical rotation of about −60° and a lower solubility (as Ca salt) than either A or C and in contrast to the latter is completely resistant to hydrolysis by testicular or bacterial hyaluronidase (Meyer and Rapport, 1951). This resistance even persists with the partly or completely desulphated compound. On decarboxylation, it yields one equivalent of CO_2 per mole of hexosamine while in the car-

(Meyer *et al.*, 1956) (Fig. 3) One of these appears to be chromato-

STERIC RELATIONSHIP BETWEEN GLUCURONIC, IDURONIC, MANNURONIC AND GULURONIC ACID

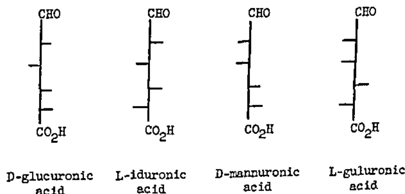


Fig 3

Steric relationship between glucuronic, iduronic, mannuronic and guluronic acid

graphically identical with D-glucuronic acid, the other with

chondroitin sulphate B, attempts were made to ascertain whether or not a mixture of polysaccharides was present. These have been inconclusive. The chemical and physical properties of B, obtained from a variety of sources and by different methods, were closely similar. Such fractions have been isolated from skin, tendon, heart valves, *ligamentum nuchae* and aorta. 'β-heparin' obtained from beef lung (Marbet and Winterstein, 1951) is identical with chondroitin sulphate B as is a fraction obtained by Smith and Gallop (1953) from hog stomach. However, the possibility still exists that chondroitin sulphate B is a mixture of at least two hyaluronidase-resistant polysaccharides.

KERATOSULPHATE

This is the only sulphated mucopolysaccharide free of uronic acid. It is composed of equimolar amounts of N-acetylglucosamine, galactose and sulphate (Meyer *et al*, 1953). Its structure is unknown and no glycosidase has been found which attacks this polysaccharide The desulphated polymer,[1] however, is hydrolysed by an enzyme obtained from *lactobacillus bifidus* with the production of monosaccharides. It is of some interest that the sulphated polymer does not cross react with blood group substances or with type XIV *pneumococcus* antiserum. The desulphated polymer precipitates type XIV *pneumococcus* antiserum comparable to group A substance of hog gastric mucosa. (We thank Dr. E. Kabat for these data.) Keratosulphate has been isolated from cornea, where it represents about 50 per cent of the total mucopolysaccharide and from growing (calf) bone, where it is only a minor component.

FRACTIONS RELATED TO HEPARIN (HEPARITIN SULPHATE)

Mucopolysaccharide fractions of a strong positive rotation and composed of equimolar amounts of glucosamine, uronic acid

[1] We are greatly [illegible]

tin sulphate combined represent the rest in approximately equal concentrations. The human aortae were of persons with moderate atherosclerosis of 45 to 84 years (average 64.1) and yielded the same polysaccharides as young bovine aorta and in strikingly similar concentrations, with the exception of hyaluronic acid which, if present at all, was well below 10 per cent of the total. The diminution of hyaluronic acid with age has also been observed in adult versus embryonal skin It is obvious from the data on skin, *ligamentum nuchae*, and aorta that chondroitin sulphate B is not a constant component of elastic tissue but that all three chondroitin sulphates are components of collagenous tissue. The histological interpretation of the differences in type and distribution of these sulphated polysaccharides in the various tissues appears, however, not possible at present.

SUMMARY

The acid mucopolysaccharides isolated from various sources have been discussed. The chemical structure of most of these is still unknown. Remarkable differences exist both in the types and in the distribution patterns of the acid polysaccharides in the different types of connective tissue.

GROUP DISCUSSION

DR. ASBOE-HANSEN asked if heparin had been detected in skin.

DR MEYER replied that in his early experiments some anti-coagulant activity had been observed in extracts of pigs' skin However, when CSA/B was carefully purified it had no heparin activity CSA/B is identical with the beta-heparin of Winterstein which had been reported to have high anti-coagulant activity

In answer to a question from Dr. Hall, DR MEYER said that no polysaccharide components other than those that he had described had been detected in *ligamentum nuchae* Moreover, he had not been able to demonstrate any mucolytic action in crude preparation of elastase CSA/B and CSA/A were prepared from *ligamentum nuchae* by alcohol fractionation of their calcium salts. In other tissues, for example calf bone, CSA/A and CSA/B could only be separated if the mixture of mucopolysaccharides is first of all treated with hyaluronidase.

DR. GILLMAN asked if one might anticipate differences in the micro-

scopic and sub-microscopic organization of elastic fibres from aorta and from skin, in view of the differences in the chondroitin sulphate and mucopolysaccharide contents of these two tissues noted by Dr. Meyer. DR. REED said that the morphology of elastin fibres from the skin is very similar to that of elastin fibres from the aorta.

DR ROBB-SMITH wondered whether the high content of CSA/A in aorta and cartilage could be correlated with the vascular and skeletal changes in experimental lathyrism and whether experimental work with beta-proprionitrile might throw some light on their metabolic behaviour

DR. MEYER did not know but said that CSA/A also occurs in cornea in which morphological or clinical changes in lathyrism have not been described. However, CSA/A constitutes a much greater fraction of the polysaccharides in aorta than in cornea. In answer to Dr. Gillman, he said he had done no work on polysaccharides in the lens

[illegible]

(*Fed Proc.*, 1956)

DR D. S JACKSON asked what Dr. Meyer's opinion was on the possibility of linkages between protein and polysaccharides in connective tissue

DR MEYER said that any bonds between hyaluronic acid and protein are comparatively weak Hyaluronic acid can be prepared chemically free of protein, and the physical properties of the material are almost identical with those of a hyaluronic acid protein complex prepared by Ogston, which contained about 30 per cent protein This does not apply to hyaluronic acid from all tissues. For example, it applies to hyaluronic acid from umbilical cord and streptococci but not to material isolated from synovial fluid Sulphated polysaccharides on the other hand are present in the tissue as protein complexes The work of Schubert has demonstrated this with CSA. The native complex and complexes prepared by adding protein to purified CSA differ in their physical properties

[illegible]

viscosity of CSA is greatly reduced by treatment with papain to digest the protein fraction. He also reported that a gelatinous fluid obtained from Heberden's nodes contained only hyaluronic acid and no protein-free or bound to the hyaluronic acid DR PARTRIDGE said he had repeated

Schubert's work. After removal of collagen by r
resin he obtained a mixture of free CSA and a co
tein. This complex could only be split by hydro
and the amino-acid analysis of the protein fraction
different from that of collagen

DR NEUBERGER and DR MEYER discussed possibl
grouping in hyaluronic acid being unreactive to pe
ably not due to the trans-configuration of the gl
also occurs in glycogen. It also seems unlikely that
bond formation or to lactone bond formation. The
steric reasons, the latter is unlikely since most of the
be esterified. The most probable reason seems to be
grouping

DR. HALL reported an observation by Dr. P. F. Llo
the electrophoretic mobility on paper of CSA/B pr
mentum nuchae is increased by about 10 per cent afte
testicular hyaluronidase

DR. MEYER could not suggest an explanation for this.

EVALUATION OF EXTRACTION METHODS FOR ACID TISSUE POLYSACCHARIDES

Olle Snellman

The chemistry and physiology of the acid polysaccharides of [illegible]

saccharides are, however, difficult to isolate and separate since the amounts occurring often are small and the different polysaccharides show similarities in structure and, from many tissues, they are even obtained as a mixture difficult to separate.

For most workers in this field, the main goal has been to prepare pure substances in order to perform structural determinations on the bonds between the different monosaccharides building up the polysaccharides. Little attention has been paid to the quantitative aspects and few attempts have been made to obtain the acids in a 'native' state, i.e. in an, as far as possible, undegraded state.

Besides the importance of getting a clear picture of the bonds and structure of the different polysaccharides, the latter questions deserve much attention. We have to know something about the state and the quantitative aspects of the different polysaccharides in the tissue since in diseases of connective tissue, commonly termed 'collagen diseases', the earliest and most profound alterations are found in the ground substance. It is with these aspects in mind we have made an investigation of the extraction methods of the acid polysaccharides.

The quantitative extraction of the acid polysaccharides is a matter of considerable difficulty and dilute sodium hydroxide is still frequently employed. This method was investigated by Jorpes (1929) using cartilage to obtain chondroitin sulphuric acid. He showed that a concentration as high as 0·5 N was necessary to extract the acid polysaccharides from cartilage at 0° C. However, the polysaccharides were obtained in a degraded state.

Many investigators have used the alkaline extraction procedure, e.g. Levene and Forge (1914), Fürth and Bruno (1937), Karrer and

Schubert's work. After removal of collagen by
resin he obtained a mixture of free CSA and a co
tein This complex could only be split by hydro
and the amino-acid analysis of the protein fractio
different from that of collagen

DR. NEUBERGER and DR. MEYER discussed possibl
grouping in hyaluronic acid being unreactive to pe
ably not due to the trans-configuration of the gl
also occurs in glycogen It also seems unlikely that
bond formation or to lactone bond formation. The
steric reasons, the latter is unlikely since most of the
be esterified. The most probable reason seems to be
grouping.

DR. HALL reported an observation by Dr. P. F. Llo
the electrophoretic mobility on paper of CSA/B pr
mentum nuchae is increased by about 10 per cent afte
testicular hyaluronidase.

DR. MEYER could not suggest an explanation for this.

EVALUATION OF EXTRACTION METHODS FOR ACID TISSUE POLYSACCHARIDES

OLLE SNELLMAN

The chemistry and physiology of the acid polysaccharides of

saccharides are, however, difficult to isolate and separate since the amounts occurring often are small and the different polysaccharides show similarities in structure and, from many tissues, they are even obtained as a mixture difficult to separate.

For most workers in this field, the main goal has been to prepare pure substances in order to perform structural determinations on the bonds between the different monosaccharides building up the polysaccharides. Little attention has been paid to the quantitative aspects and few attempts have been made to obtain the acids in a 'native' state, i.e. in an, as far as possible, undegraded state.

Besides the importance of getting a clear picture of the bonds and structure of the different polysaccharides, the latter questions deserve much attention. We have to know something about the state and the quantitative aspects of the different polysaccharides in the tissue since in diseases of connective tissue, commonly termed 'collagen diseases', the earliest and most profound alterations are found in the ground substance. It is with these aspects in mind we have made an investigation of the extraction methods of the acid polysaccharides.

The quantitative extraction of the acid polysaccharides is a matter of considerable difficulty and dilute sodium hydroxide is still frequently employed This method was investigated by Jorpes (1929) using cartilage to obtain chondroitin sulphuric acid. He showed that a concentration as high as 0 5 N was necessary to extract the acid polysaccharides from cartilage at 0° C. However, the polysaccharides were obtained in a degraded state

Many investigators have used the alkaline extraction procedure, e.g. Levene and Forge (1914), Fürth and Bruno (1937), Karrer and

Mayer (1937), Bray, Gregory and Stacey (1944), Meyer, Odier and Siegrist (1948), Strandberg (1950) and Leon and Deiss (1954).

Meyer and Smyth (1937) suggested the use of strong solutions of calcium chloride and obtained good yields of chondroitin sulphate by the extraction of dried cartilage powder with 10 per cent calcium chloride solution at neutral reaction. Blix and Snellman (1945) used calcium chloride extraction and were careful to avoid acid or alkaline solutions during the subsequent separation procedures. The aim was to obtain, as nearly as possible, the polysaccharides in an undegraded state.

with this method and, by subsequent extractions using alkaline solutions, 0.80 g. could be removed. The tissue residues after this extraction were, however, not investigated.

Hyaluronic acid generally occurs together with sulphated polysaccharides. The extraction procedures of this acid vary somewhat among different workers but, principally, the methods are quite similar. Most of the principles used originate from the work of K. Meyer, the first to isolate hyaluronic acid.

Fluids such as synovial fluid, tumour fluid and vitreous humor are

prior to the precipitation in order to increase the amount of polysaccharide precipitated in a mucin-like clot. From the clot the hyaluronic acid is taken up by extraction with potassium acetate at pH 9. Proteins are then removed from the solution by shaking with chloroform-amyl alcohol (Sevag, 1934) and further contaminations are removed by Lloyd's reagent at pH 4.

Meyer (1948) prepared sodium hyaluronate of high viscosity by extracting homogenized human umbilical cords with 2 per cent phenol and clotting the diluted mixture with acetic acid. The pro-

then under similar conditions with trypsin at pH 7.4 After filtration the proteins have been eliminated from the solution by shaking with chloroform-amyl alcohol.

In order to isolate hyaluronic acid and chondroitin sulphuric acid from skin, alkaline solutions have been widely used (Meyer and Chaffee (1941), Pearce and Watson (1949), Schiller, Mathews *et al* (1954)). [illegible] proteins a [illegible] difficulties [illegible] alkaline solutions are not used.

Schiller *et al.* (1954) treated the skin with a 2 per cent alkaline solution for 24 hours They neutralized and dialysed the solution and incubated with trypsin for 5 days. Afterwards, they precipitated with trichloroacetic acid, centrifuged and dialysed again The solution was precipitated with ethanol in the presence of 1 per cent potassium acetate. On the precipitate, a slab electrophoresis was made according to Gardell *et al.* (1950) Hereby they could separate the hyaluronic acid and chondroitin sulphuric acid.

Gardell (1952) has boiled the tissue and afterwards digested it with an extract of pancreas for many days and purified the product with 10 volumes of ethanol and later with Lloyd's reagent. A slab electrophoresis was then performed.

In the case of heparin, the method described by Charles and Scott (1933) appears to have been generally adopted Here the tissue is first autolysed for 24 hours at room temperature, since higher yields are then obtained The tissue is extracted at 50° C for one hour with [illegible]

the heparin-protein complex. The precipitate is washed by different means and then dissolved in alkaline solution, and the proteins are subjected to trypsin digestion.

[illegible]

Homan and Lens (1948), who have worked out a tedious procedure, which is said to cause not so heavy losses.

In this short description we have only reviewed the main principles used to extract the three groups of acid polysaccharides. Some methods giving low yields have been omitted.

Besides the various extraction methods, quite another method ha been used by Consden *et al.* (1953) to obtain knowledge about th saccharides occurring in the tissue. Consden autoclaves smalle pieces of the tissue in water in order to remove the collagen and the he hydrolyses and determines the different monosaccharides in th hydrolysate. This method can give valuable information abou the basic carbohydrates that occur in the tissue but the method ha only a limited value since it does not give any informations abou the polysaccharides to which the carbohydrates belong.

methods extract the polysaccharides as we are lacking careful analyses on that point. Furthermore, all investigators extracting hyaluronic acid and chondroitin sulphuric acid have completely missed the heparin. It seems either to have been lost or obtained as a contamination in the fraction of the other polysaccharides, difficult to separate from them. Thus Sylvén and Malmgren (1952) purify hyaluronate by precipitating sulphate-containing polysaccharides with Azure A. Apparently, many authors have not succeeded in preparing sulphate free hyaluronic acid.

In a great number of investigations, alkaline solutions have been used to dissolve the tissue. I have the impression that the best yields are obtained by this method, and if any losses are obtained, they mostly come in the subsequent purifying processes.

However, the use of alkaline solutions involves the danger of degrading effects on the polysaccharides, and when this method is used nothing can be said about the 'native' state. The experience

extracted tissues do not have these disadvantages, at least to any high degree. But they are not quantitative and mixtures have been obtained difficult to separate. Thus, Hadidian and Pirie (1948) report in their fractions of hyaluronic acid, sulphur contents of 0.5-1 per cent, as usual. Most investigators have only, as it seems, been interested in a fairly good yield of hyaluronate not looking for other polysaccharides occurring.

The method of Einbinder and Schubert is also a mild method and may give an undegraded product. It is worked out for cartilage and,

to obtain good yields, the material is stored for a long time. What this treatment does to the product we don't know. Evidently according to the investigations of Malmgren and Sylvén (1952), the method is not quantitative.

Nearly every investigator has used his own purification scheme, often only a modification of some principles used in all the methods.

Many have used precipitation reactions going from very alkaline solutions to very acid solutions. These methods involve considerable [illegible]

Kaolin has been used in several cases as an adsorbent but the proteins are not effectively removed by this mean and, if the extracts are too heavily treated by kaolin, losses of polysaccharide will occur Nor is phenol or glacial acetic acid effective.

The method of shaking the extract with chloroform-amyl alcohol has often been used in order to separate protein and polysaccharide. The method involves a denaturation of proteins If a quantitative separation is wanted, the treatment must be performed [illegible]

back again into the water phase. Some contaminations remain in the solution. It seems better to purify afterwards with $Zn(OH)_2$ than by Lloyd's reagent

Concerning the two types of polysaccharides hyaluronic acid and chondroitin sulphuric acid, the best method to release them from their protein associations seems to be by proteolytic enzyme digestion. The fission products are then comparatively easily removed.

Of the proteolytic enzymes used, pepsin has too low a pH optimum. Crude pepsin is also, according to Meyer (1945), often contaminated with a polysaccharide which might render this treatment dangerous Crystalline trypsin or chymotrypsin can be used, but are said to be less effective (Gardell, 1952). Gardell uses a crude glycerol extract from the pancreas and the intestine This is, however, contaminated by polysaccharides and contains at least one enzyme which splits glycogen. [illegible]

After the enzyme digestion, a preliminary purification can be achieved by dialysis and precipitation by organic solvents.

In many investigations the separation of hyaluronic acid and chondroitin sulphuric acid has been performed by precipitation of the calcium salts with ethanol. Precipitation with a 20 per cent ethanol solution is said to separate the polysaccharides. In spite of much use, the method has not been thoroughly investigated and, in the rough manner it has been used, no very good separations have been achieved. Thus Meyer and Chaffee (1941) use pneumococcal hyaluronidase in order to purify the chondroitin sulphuric acid further. Any kind of electrophoretic separation, as used by Gardell and Schiller *et al.*, is superior.

[illegible]

this tissue seems to be especially troublesome.

The first step is a thorough defatting procedure, which we have found to be very important in order to obtain good yields and which seems to make the alkali treatment unnecessary. Probably it is the fatty material which prevents the extraction solution from coming in an intimate contact with the material in many cases. An outstanding example in this respect is the pleural tissue from which the heparin has been reported not to be extractable (Wegelius, 1956). However, we have found it possible to extract the heparin from this tissue after an extensive defatting procedure. Generally, we dry the frozen minced tissue at first in acetone and then defat it in a Soxhlet apparatus with acetone-ether (1:5). After this procedure, the tissue is minced again.

The next step is to extract the heparin with potassium thiocyanate (Snellman, Jensen and Sylvén, 1948). One point may be stressed here. In general tissue homogenates will very rapidly become acid and therefore the solutions must be buffered and the pH controlled. At slightly acid pH the heparin associates with proteins in the mixture, and that will cause trouble and even losses of substance. If the precaution is made to hold the pH at about 7.5 the whole time the proteins can easily be removed and, as far as we can judge, the heparin can be taken out and isolated quantitatively.

[illegible] nic acids

h of the

polyu-

ronic acids remains in the residues which are extracted with a calcium [illegible] ments.

However, after these treatments residues are obtained. Alkali treatments of these residues at room temperature do not release any hexuronic acid but still there are some carbohydrates left undissolved. The method of Consden has been used on this material but no hexuronic acid could be detected, so it seems that no hexuronic acid-containing polysaccharide has been left as far as can be judged by this method

Having thus obtained the different extracts containing the polysaccharides, the latter must be isolated and purified.

In the case of heparin, the solution has been precipitated with one volume of acetone and dissolved again in a small volume of buffer solution, and then reprecipitated with 60 per cent ethanol. By this procedure, much of the proteins are removed without any appreciable loss of heparin The precipitate is then dissolved again in a suitable amount of phosphate buffer at pH 7.2 and a zone electrophoresis is performed.

We have found it most suitable to make the zone electrophoresis according to Flodin and Porath (1954) using an ethanolysed cellulose as supporting medium This cellulose is easier to pack than Hyflo Super-Cel and does not adsorb the acid polysaccharides. With such a column even greater amounts of material can be separated in an easy manner and the column has a good separation power.

In the case of heparin, all substances of low electric charge, such as [illegible] vestigated. By an ethanol precipitation they were separated and heparin free of amino acids and nucleotide material was obtained In one experiment 81 mg of heparin, as determined by activity measurements, was found in the sample and 80 mg., determined in the same manner, was found in the final product, when liver capsule [illegible]

the zone electrophoresis.

From the discussion given we may conclude that in most cases it is unnecessary to begin with alkaline solutions. Such treatments do not seem superior to the other milder methods as a means of extraction for the acid mucopolysaccharides. In our experiments we have tried to investigate all solutions with the carbazole test of Dische (1947) in a qualitative manner to be sure that no hexuronic acid had been lost in the purification procedures. It thus seems that the hexuronic acids can be extracted and isolated in an as near as possible quantitative way and in a state which seems to be undegraded. If any further separation in components of the different substances or any other impurities than amino acids can occur has as yet not been investigated.

The carbohydrates in the residues have not as yet been further investigated. They must be very closely connected to proteins difficult to remove. It is possible that harder means must be used to isolate them These residue carbohydrates may, from other points of view, have a great interest but they do not seem to contain any hexuronic acids.

[illegible] supported by grants from [illegible] edish

GROUP DISCUSSION

Dr. D. S. Jackson mentioned the use of quaternary ammonium salts for the specific precipitation of acid polysaccharides (Dr. G. E. Scott, personal communication) Complete separation of heparin, CSA and hyaluronic acid from each and from neutral polysaccharides can be achieved simply and rapidly.

Dr. Meyer pointed out that the Carbazole method might give erroneous results for uronic acid content. For example, iduronic acid and mannuronic acid give much less coloration than glucuronic acid.

In reply to Dr. Consden, Dr Snellman said that the Carbazole method was the only one he had used for determination of uronic acid.

COMBINATIONS *IN VITRO* COLLAGÈNE-MUCOPOLYSACCHARIDES ET MODIFICATIONS APPORTÉES À CES COMBINAISONS PAR DES SELS ET DES POLYOSIDES BACTÉRIENS[1]

ALBERT DELAUNAY ET SUZANNE BAZIN

INTRODUCTION

On sait depuis plusieurs années que l'addition de sels ou de mucopolysaccharides à une solution de collagène donne souvent lieu à une précipitation. Tout récemment (Delaunay, Bazin et [illegible]

Dans le travail ici présenté, nous rapporterons ce que nous avons constaté en étudiant les principaux caractères des combinaisons collagène-mucopolysaccharides et collagène-polyosides bactériens. Nous dirons aussi, sur la foi de nos observations, comment une combinaison normale collagène-mucopolysaccharide peut être modifiée quand elle doit prendre place en présence de sels ou de polyosides bactériens

PARTIE EXPERIMENTALE

A. – *Matériel utilisé*

1. Collagène. Nous avons utilisé, pour toutes nos expériences, un collagène acido-soluble le collagène A (Nageotte, 1927). Nos [illegible]

4,3 à 4,5).

2. Mucopolysaccharides (M P.). (*a*) Héparine. Ce produit purifié, provenait de la maison Hoffmann-Laroche. (*b*) Acide chondroïtine sulfurique. L'échantillon dont nous nous sommes servi nous avait été obligeamment fourni par le Pr. Fauré-Frémiet. Il avait été extrait du cartilage de veau selon les techniques de P. A Levène. Avant usage, nous avons tenté de le *purifier encore par 3 prépicipations* successives (au moyen de 3 volumes d'alcool).

[1] Collagen-mucopolysaccharide combinations *in vitro* and how they are affected by bacterial polysaccharides and salts

3. Polyosides bactériens. (*a*) et (*b*) Lipopolysaccharide (endotoxine) et polyoside typhiques. Ils ont été isolés, puis purifiés, selon [illegible]

[illegible] fugation, le liquide surnageant est dialysé puis concentré 10 fois. Cette préparation est soumise à des précipitations fractionnées par l'alcool, enfin desséchée sous vide. (*d*) Polyoside Cx. Pour obtenir celui-ci, nous avons suivi une méthode calquée sur celle de Anderson et McCarty (1951). Des pneumocoques rough du type II sont détruits rapidement par le désoxycholate de sodium. Les protéines microbiennes sont coagulées par la chaleur en milieu acétique. La fraction polyosidique est précipitée par l'alcool puis séparée des acides nucléiques au moyen de précipitations fractionnées par l'alcool en présence de Cl_2Ca.

4. Sels: chlorure et sulfate de sodium. Ces sels se trouvaient en solution dans l'eau distillée.

B. — *Technique opératoire*

1. A divers échantillons de notre collagène A a été ajoutée, sou volume égal, soit une solution de M.P., soit une solution de polyosides bactériens, soit une solution saline, soit, enfin, une solution contenant en mélange un M.P. et un sel ou un M.P. et un polyoside bactérien.

Les sels quand ils ont été utilisés isolément, l'ont été à différentes concentrations: pour le ClNa, de 0,85 à 0,0213 M, pour le SO_4Na_2, de 0,427 à 0,00427 M. En ce cas, nous avons laissé aux solutions leur pH naturel. Quand, avant l'expérience, les sels avaient été mélangés à un M.P., le pH du mélange était toujours ajusté à 4,3.

M.P. ou polyosides bactériens, employés isolément, ont été, de [illegible] collagène à diverses concentrations et à différents [illegible] les polyosides se trouvaient à doses variables, les M.P. à une seule dose qui était la dose précipitante optimum. Nous avons pris l'habitude de désigner sous ce nom la dose de M.P. (ou de polyosides) qui, ajoutée à une quantité donnée de collagène A, conduit à la formation d'un précipité qui contient tout le collagène présent et la majeure partie, sinon la totalité, du produit ajouté. L'absence de

collagène dans le liquide surnageant est démontrée par la négativité des réactions caractéristiques de l'hydroxyproline, l'absence des [illegible]

2. Après addition au collagène A des diverses solutions définies ci-dessus, les tubes sont légèrement agités puis conservés pendant 3 heures à 18° C. A ce moment, leur contenu est examiné: on note s'il y a eu, ou non, précipitation. En cas de résultat positif, l'importance du précipité est estimée et son aspect macroscopique étudié.

3. Ceci fait, les précipités formés sont recueillis par centrifugation puis repris par de l'eau distillée amenée à différents pH Nous avons déterminé de la sorte les marges de pH en dehors desquelles il y a dissolution des précipités

Certains précipités, remis après lavage dans de l'eau amenée au pH du milieu où avait eu lieu la précipitation, ont été soumis à l'action du chauffage et du Cl_2Ca. Le chauffage, d'une durée de 3 minutes, a eu lieu à des températures variables. Nous avons noté la température la plus basse qui est suivie d'une contraction ou d'une dissolution du précipité. En ce qui concerne le chlorure de calcium, nous avons déterminé, dans chaque cas, la concentration limite qui est capable, à diverses températures, de dissoudre complètement le précipité examiné.

RESULTATS

A. – *Après addition au collagène d'héparine et d'acide chondroïtine-sulfurique (A.Ch.S)*

1. Principaux caractères des précipitations observées. Le lecteur trouvera, reporté dans le tableau I, l'essentiel de nos résultats

TABLEAU I

CARACTÈRES DE LA PRÉCIPITATION OBTENUE APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, D'HÉPARINE OU D'ACIDE CHONDROÏTINE-SULFURIQUE

Nature de la substance utilisée pour la précipitation du collagène	*Limite d'activité de la substance précipitante*	*Dose précipitante optimum*	*Marges de pH entre lesquelles peut se produire une précipitation*	*pH du milieu où la précipitation paraît être optimum*
Héparine	0,02 mg	0,10 mg	1,5-8	4,3
Acide chondroïtine sulfurique	0,05 mg	0,20 mg	2-8	4,3

supérieure à 5 mg/ml n'a donné, dans les mêmes conditions qu'un faible précipité.

2. Principaux caractères des précipités obtenus. Prière de se reporter, pour les connaître, au tableau II.

TABLEAU II

CARACTÈRES DES PRÉCIPITÉS OBTENUS APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, D'HÉPARINE OU D'ACIDE CHONDROÏTINE-SULFURIQUE

Nature de la substance utilisée pour la précipitation du collagène*	*Aspect du précipité obtenu*	*Température de contraction*	*Marges de pH en dehors desquelles le précipité se dissout*	*Concentration limite (M) de Cl_2Ca capable de dissoudre complètement le précipité à 18° C*
Héparine	Fibres longues et agrégées	48° C.	1,2-11	0,60
Acide chondroïtine-sulfurique	Fibres longues et agrégées	48° C	1,5-11	0,06

* A dose précipitante optimum

Ajoutons une précision. Des concentrations de Cl_2Ca, inférieures

B. — *Après addition au collagène de divers polyosides bactériens*

1. Principaux caractères des précipitations observées. Ils sont indiqués dans le tableau III.

TABLEAU III

CARACTÈRES DE LA PRÉCIPITATION OBTENUE APRÈS ADDITION, A UNE SOLUTION DE COLLAGÈNE DE DIVERS POLYOSIDES BACTÉRIENS

Nature de la substance utilisée pour la précipitation du collagène	*Limite d'activité de la substance précipitante*	*Dose précipitante optimum*	*Marges de pH entre lesquelles peut se produire une précipitation*	*pH du milieu où la précipitation paraît être optimum*
Lipopolysaccharide (endotoxine) typhique	0,25 mg	3 mg	1,5-10,5	4,3
Polyoside typhique	0,50 mg	1 mg	5 -10	7,2
Polyoside staphylococcique	0,10 mg	0,50 mg	3 -7,2	4,3
Polyoside pneumococcique (Cx)	0,20 mg	0,50 mg	2,5-7,?	4,3

2. Principaux caractères des précipités obtenus. On les trouvera, exposés, dans le tableau IV.

TABLEAU IV

CARACTÈRES DES PRÉCIPITÉS OBTENUS APRÈS ADDITION, A UNE SOLUTION DE COLLAGÈNE A, DE DIVERS POLYOSIDES BACTÉRIENS

Nature de la substance utilisée pour la précipitation du collagène*	*Aspect du précipité obtenu*	*Température de dissolution*	*Marges de pH en dehors desquelles le précipité se dissout*	*Concentration limite (M) de Cl_2Ca capable de dissoudre complètement le précipité à 18° C*
Lipopolysaccharide (endotoxine) typhique	Fibres très courtes et non agrégées	56° C	1-12	0,01
Polyoside typhique	Fibres mal formées. Précipité gélatineux	52° C	5-12	0,01
Polyoside staphylococcique	Fibres longues et agrégées	54° C	2-11	0,05
Polyoside pneumococcique (Cx)	Fibres courtes et non agrégées	54° C	2,2-10,5	0,20

* A dose précipitante optimum.

Remarque. Des concentrations de Cl_2Ca inférieures de 2 à 3 fois aux concentrations limites indiquées ci-dessus (qui sont valables pour des expériences faites à 18° C.) sont capables d'abaisser la température de dissolution des précipités de 10 à 3° C.

C. — *Après addition au collagène de sels: ClNa et SO_4Na_2*

Le lecteur trouvera, indiquées dans le tableau V, à côté de la nature du sel utilisé, les concentrations moléculaires qui sont, ou non, précipitantes (+++ indique la formation d'un précipité abondant). Il connaîtra aussi le pH du mélange sel+collagène (le sel étant utilisé à la dose précipitante limite).

TABLEAU V

Nature du sel	*pH du mélange sel+ collagène*	*Concentration moléculaire en sel*				
		0,00427	0,0427	0,085	0,213	0,427
ClNa	4,65	o	o	o	o	+++
SO_4Na_2	5,2	o	o	o	+++	+++

[illegible]

la solution saline et placés dans l'eau distillée s'y dissolvent (cela, quel que soit le pH du milieu)

D. — *Après addition au collagène d'un mélange de M.P. (Héparine ou A.Ch.S)+sels (ClNa ou SO_4Na_2)*

Rappelons que, dans ces mélanges, les M.P. se trouvaient à dose précipitante optimum (soit, pour l'héparine, 0,10 mg. et pour l'A.Ch.S., 0,20 mg.) et les sels à doses normalement précipitantes ou non.

1. Héparine+doses non précipitantes de ClNa. Précipitation (type héparine) normale en présence de 0,0213 M, faible en présence de 0,0427 M; pas de précipitation en présence de concentrations salines comprises entre 0,085 M et 0,213 M.

2 Héparine+dose précipitante de ClNa (0,427 M); pas de précipitation.

3. Héparine+doses non précipitantes de SO_4Na_2: Précipitation (type héparine) normale en présence de 0,0085 M, faible en présence de 0,0213 M; pas de précipitation en présence de 0,085 M.

4. Héparine+doses précipitantes de SO_4Na_2. Pas de précipitation en présence de 0,213 M; précipitation (type sel) peu abondante en présence de 0,427 M.

5. A.Ch.S +doses non précipitantes de ClNa: Précipitation (type A.Ch S.) normale en présence de 0,085 M. Obtention d'un précipité abondant mais de caractères particuliers en présence de 0,213 M. Ce précipité est particulier parce qu'il ne se contracte pas à 48° C. comme un précipité collagène-A Ch.S normal et qu'il ne se dissout pas dans l'eau distillée comme un précipité collagène-ClNa normal; il se dissout à 56° C.

6. A.Ch S.+dose précipitante de ClNa (0,427 M). Précipitation (type sel) normale

7. A.Ch.S.+doses non précipitantes de SO_4Na_2. Précipitation (type A Ch S.) normale en présence de 0,0427 M et de concentrations salines plus faibles. Obtention d'un précipité abondant mais de caractères particuliers en présence de 0,085 M. Ce précipité, particulier parce qu'il ne se contracte pas à 48° C et qu'il ne se dissout pas dans l'eau distillée, se dissout à 52° C.

8. A Ch S.+doses précipitantes de SO_4Na_2: Obtention d'un précipité peu abondant et de caractères particuliers (particuliers pour les mêmes raisons que celles données ci-dessus. cf paragraphes 5 et 7) en présence de 0,213 M. En présence de 0,427 M, précipitation (type sel) peu abondante.

E. — *Après addition au collagène d'un mélange M.P (héparine ou A Ch.S.)+polyosides bactériens*

Le lecteur trouvera dans le tableau VI l'essentiel des résultats que nous avons recueillis quand, dans le mélange, mucopolysaccharide et polyoside bactérien étaient utilisés, tous deux, à la dose précipitante optimum. Il trouvera aussi, dans les lignes supérieures de ce tableau, le rappel des faits que l'on constate quand on utilise isolément, à cette même dose, mucopolysaccharides et polyosides, une étude comparative sera de la sorte grandement facilitée

au

bactériens, les premiers étant toujours à dose précipitante optimum, les seconds au contraire étant tantôt à dose forte (c'est-à-dire supérieure), tantôt à dose faible (c'est-à-dire inférieure à la dose précipitante optimum).

1. Héparine+lipopolysaccharide typhique. (*a*) à dose forte

TABLEAU VI

CARACTÈRES DES PRÉCIPITÉS OBTENUS APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, DE MUCOPOLYSACCHARIDES, DE POLYOSIDES BACTÉRIENS OU D'UN MÉLANGE MUCOPOLYSACCHARIDE +POLYOSIDE BACTÉRIEN (TOUS CES CORPS ÉTANT UTILISÉS À LA DOSE PRÉCIPITANTE OPTIMUM)

Nature des substances précipitantes utilisées	*Importance du précipité obtenu*	*Aspect du précipité obtenu*	*Action de la chaleur sur le précipité*	
			Température de contraction	*Température de dissolution*
Héparine	+++	Fibres longues, agrégées	48° C	
Acide chondroïtine-sulfurique	+++	Fibres longues, agrégées	48° C.	
Lipopolysaccharide (endotoxine) typhique	+++	Fibres très courtes, non agrégées		56° C.
Polyoside typhique*	+++	Fibres mal formées (précipité gélatineux)		52° C.
Polyoside staphylococcique	+++	Fibres longues, agrégées		54° C.
Polyoside pneumococcique (Cx)	+++	Fibres courtes, non agrégées		54° C
Héparine+lipopolysaccharide (endotoxine) typhique	++±	Fibres courtes, non agrégées		56° C.
Héparine+polyoside typhique	+±	Fibres longues (précipité non gélatineux)	50° C	
Héparine+polyoside staphylococcique	o			
Héparine+Cx	+++	Fibres, longues agrégées	48° C	
Acide chondroïtine-sulfurique+lipopolysaccharide (endotoxine) typhique	++±	Fibres courtes, non agrégées		56° C
Acide chondroïtine-sulfurique+polyoside typhique	++	Fibres longues (précipité non gélatineux)	50° C	
Acide chondroïtine-sulfurique+polyoside staphylococcique	+++	Fibres longues, agrégées	48° C	
Acide chondroïtine-sulfurique+Cx	+++	Fibres longues, agrégées	48° C.	

* Observations faites exceptionnellement, non à pH 4,3, mais à pH 7

(5 mg./ml.): obtention d'un précipité (type endotoxine) normal.

[illegible]

se fait, non en une seule masse, mais en petits grains.

2. Héparine+polyoside typhique. (*a*) à dose forte (2 mg./ml.): précipitation anormalement faible; le précipité se contracte à 50° C. (*b*) à dose faible (0,25 mg./ml.): précipitation (type héparine) normale.

3. Héparine+polyoside staphylococcique. (*a*) à dose forte (2 mg./ml.) et (*b*) à dose faible (0,20 mg./ml.): pas de précipitation.

4. Héparine+polyoside pneumococcique Cx. (*a*) à dose forte (1 mg./ml.): obtention d'un précipité type héparine mais qui se contracte à 50° C. (*b*) à dose faible (0,2 mg./ml.). obtention d'un précipité (type héparine) normal.

5. A.Ch.S.+lipopolysaccharide typhique. (*a*) à dose forte (5 mg./ml.): obtention d'un précipité (type endotoxine) normal. (*b*) à dose faible (1 mg./ml.): obtention d'un précipité (type A.Ch.S.) normal.

6. A.Ch.S.+polyoside typhique (*a*) à dose forte (2 mg./ml.). obtention d'un précipité type A.Ch.S. mais qui se contracte à 50° C. (*b*) à dose faible (0,25 mg./ml.)· obtention d'un précipité (type A.Ch.S.) normal.

7. A.Ch.S.+polyoside staphylococcique. (*a*) à dose forte (2 mg./ml.) et (*b*) à dose faible (0,20 mg./ml.): obtention d'un précipité (type A.Ch.S.) normal.

8. A.Ch.S.+polyoside pneumococcique Cx. (*a*) à dose forte (1 mg./ml.): obtention d'un précipité type A.Ch.S. mais qui se contracte à 50° C. (*b*) à dose faible (0,2 mg./ml.); obtention d'un précipité (type A.Ch.S.) normal.

DISCUSSION

1. On savait déjà que des mucopolysaccharides comme l'héparine et l'A.Ch.S sont capables de précipiter tous deux un collagène soluble en donnant naissance à de belles fibrilles A la lumière de nos
-I -

M.r. ajoute (ceci étant démontré par le double epuisement du liquide surnageant).

– Dans les précipités, il y a non seulement présence mais encore combinaison des deux principes chimiques. Comme preuve, nous donnerons les résultats de l'expérience suivante. A des échantillons d'une même sol…
d'l
pr
dosage de l'hexosamine dans 1 mg. de précipité sec 26 µg 7, 27 µg 1;

27 µg 8, 27 µg 3. Quatre chiffres aussi voisins n'auraient pu être *trouvés si une véritable* combinaison chimique n'était pas en cause (dans le collagène A précipité par le ClNa, pas d'hexosamine décelable par la méthode que nous avons utilisée).

– Soumis à l'action de la chaleur, les précipités collagène-héparine ou collagène A.Ch.S. se comportent de la même façon puisque tous deux se contractent à 48° C. En revanche, le premier est plus aisément dissociable par le Cl_2Ca; il serait donc moins stable.

2. Des polyosides bactériens comme le lipopolysaccharide typhique, les polyosides typhique et staphylococcique, enfin le polyoside pneumococcique Cx peuvent, au même titre que les M P. *mentionnés plus haut, précipiter in vitro* le collagène A *Cette fois encore,* à une exception près, nous avons insisté sur cette exception qui con-
travail précédent (Bazin, Del-
e les précipitations tradui-
Combinaisons, toutefois,
très particulières puisque, non seulement elles diffèrent totalement des combinaisons collagène-M.P., mais qu'elles diffèrent encore profondément entre elles. Ainsi:

– La dose précipitante optimum varie nettement d'un polyoside bactérien à l'autre; en tout cas, cependant, nous l'avons toujours trouvée supérieure, et souvent très supérieure, aux doses correspondantes pour l'héparine et l'A.Ch.S.

– Les marges de pH qui permettent une précipitation sont, de leur côté, non moins variables.

– Variabilité encore, en ce qui concerne l'aspect et les propriétés des précipités obtenus. On observe la formation, tantôt de belles fibrilles, tantôt d'un simple dépôt gélatineux. Mais quels qu'ils
soi
Pa

polyosides bactériens sont moins solides que les combinaisons collagène-M.P.

utilisés à doses précipitantes *et même à doses non précipitantes.* Cette action inhibitrice est particulièrement nette dans le cas de l'héparine.

Pareille observation mérite sans doute d'être rapprochée de

certaines, autres recueillies dans des domaines voisins. Ainsi, Blumberg, Oster et Meyer (1955) ont signalé que, mis en présence de faibles concentrations de ClNa, l'acide hyaluronique donne des agrégats particulaires, et que ces agrégats, au contraire, se dissocient quand le ClNa présent dans le milieu atteint une concentration phy- [illegible] centration de 5%, inhibe fortement la combinaison héparine protamine (en présence de ClNa, 5 fois moins d'héparine se trouvent fixés). D'après S. S. Cohen (1942), la précipitation d'une nucléoprotéine (il s'agissait en l'occurrence du virus de la «tomato bushy stunt») par l'héparine, l'A.Ch.S. et l'acide hyaluronique, est inhibée par 0,1 M de ClNa; elle reprend place, cependant, en présence d'une plus grande quantité de sel. Ajoutons enfin que, selon J Badin et [illegible] inhibée totalement par 0,2 M du même corps

4. Les combinaisons collagène-M.P. (héparine ou A.Ch S.) cessent aussi de se faire normalement *in vitro* quand, dans le milieu, se trouve un polyoside bactérien comme le lipopolysaccharide typhique, les polyosides typhique et staphylococcique, enfin le polyoside pneumococcique Cx.

Les combinaisons collagène-héparine sont surtout troublées quand, dans le milieu, se trouve un lipopolysaccharide typhique ou un polyoside staphylococcique Parfois, on ne note aucune précipitation Si elle se produit, elle est le plus souvent peu abondante; encore le précipité formé est-il en général du type collagène-polyoside bactérien (ex: collagène-lipopolysaccharide typhique).

Les combinaisons collagène-A.Ch.S. sont, elles aussi, troublées surtout par la présence du lipopolysaccharide typhique et du polyoside staphylococcique Dans l'ensemble, toutefois, elles sont troublées moins nettement que les précédentes, ce qui étonne d'ailleurs un peu, attendu que, d'après l'action du Cl_2Ca, on serait tenté de les croire normalement moins stables

5. Motifs d'intérêt des observations mentionnées ci-dessus. Ils sont, à notre avis, divers. Nous les résumerons ainsi.

(*a*) D'assez nombreux auteurs pensent à l'heure actuelle que des mucopolysaccharides sont un des éléments du ciment qui unit, dans

la fibre conjonctive, les fibrilles et les filaments. Nous ne pouvon pas dire que nos recherches personnelles apportent un argumen supplémentaire en faveur de cette conception. Nous croyons pour tant qu'en mettant en évidence la production *in vitro* de véritabl combinaisons chimiques entre collagène et M.P., elles offrent l grand avantage de souligner l'affinité extraordinaire que possèden ces substances les unes pour les autres. Que celles-ci se « lient » dan [illegible]

les molécules de M.P. Mais pourquoi ces liaisons seraient-elles défectueuses ou, si l'on veut dangereuses? Les faits expérimentaux que nous avons relatés ci-dessus à propos des sels et des polyosides bactériens apportent une réponse au moins plausible. Ces liaisons deviendraient défectueuses parce que se trouveraient à un moment donné, dans la trame conjonctive, des éléments qui sont capables, eux aussi de contracter des liaisons avec le collagène ou les M.P. Se ferait jour ainsi une action compétitive entre plusieurs *substances*, d'où, en dernière analyse, formation d'un ciment mal lié. Au nombre de ces substances, nous n'hésitons pas, en nous fondant sur nos résultats *in vitro*, à ranger des sels (ils seraient présents, localement, à des concentrations trop faibles ou trop fortes) et des principes bactériens divers (par exemple, polyosides).

(*c*) Il est toujours dangereux d'extrapoler et de vouloir imaginer ce qui peut se passer *in vivo* d'après ce qu'on voit *in vitro*. Nous le savons mieux que quiconque. Il n'en reste pas moins vrai qu'au point atteint par nos recherches, nous faisons à l'hypothèse mentionnée le plus large crédit.

RESUMÉ ET CONCLUSION

Les auteurs ont étudié les combinaisons qui se forment *in vitro* entre le collagène A d'une part, des mucopolysaccharides (héparine, acide chondroïtine-sulfurique) ou des polyosides bactériens (lipopolysaccharide typhique, polyosides typhique et staphylococcique, polyoside pneumococcique Cx) de l'autre.

Ils ont ensuite montré comment des combinaisons collagène-

mucopolysaccharides peuvent être troublées par la présence dans le milieu de sels (ClNa, SO_4Na_2) ou de polyosides bactériens.

En conclusion, ils ont émis une hypothèse d'après laquelle certaines « maladies du collagène » pourraient résulter simplement de liaisons devenues défectueuses entre la protéine collagène et les molécules de M P., ces liaisons défectueuses tenant à l'entrée en jeu intempestive (action compétitive) de sels ou de principes bactériens.

SUMMARY

1. When to a solution of collagen A a solution of heparin or chondroitin sulphuric acid is added, a precipitate forms This fact has been known for several years, by electron microscopists in particular, but has never been the object of a systematic study.

We have determined, for the two mucopolysaccharides quoted, the minimum precipitating dose, the optimum precipitating dose, the limits of pH between which precipitation occurs, the pH of the medium for apparent optimum precipitation, etc. We have also studied systematically the principal characters of the precipitates obtained (appearance, temperature of contraction, etc.) We were also able to show that the precipitates are the result of a true chemical combination between the collagen and the precipitating substance.

2. A precipitate may also be obtained when bacterial polysaccharides are added to a solution of collagen A We have discovered this fact by using typhoid *lipopolysaccharide (endotoxin)*, *typhic* and staphylococcal polysaccharides, or the Cx polysaccharide extracted from pneumococcus (rough). The precipitates obtained in this way differ not only from those given by chondroitin sulphuric acid and by heparin but also they differ from each other. For each bacterial polysaccharide we have defined the conditions for precipitation and the nature of the precipitates formed.

3. Having established these facts, we have studied what happened when heparin or chondroitin sulphuric acid was added to collagen A in the presence of salts or of a bacterial polysaccharide. The results are as follows:

(*a*) In the presence of salts (NaCl, sodium sulphate) we obtained either a very faint precipitate having different properties or no precipitate at all. The variability of the results depended on the nature of the mucopolysaccharide used and on the nature and amount of the salts present in the medium.

(*b*) The combinations collagen-heparin or collagen-chondroitin sulfuric acid are also altered when they take place in the presence of a bacterial polysaccharide. *Precipitation may be inhibited. More* often precipitation occurs but is of an unknown type.

4. The authors have attempted to determine the significance of *these observations both in biochemistry and pathology.*

GROUP DISCUSSION

[illegible]

bine stoichiometrically with collagen A. [illegible]

tween collagen and mucopolysaccharides.

Dr D. S Jackson referred to experiments by Dr. Bungenberg de Jong working at Utrecht. This worker, up to the present date, he said, had studied the interaction of a large number of polyelectrolytes and neutral polymers with every possible combination of anions and cations and had evoked a theoretical explanation for these phenomena Dr. Delaunay's observations could be a specific example of the general phenomenon of colloid interaction

Dr. Baló remarked that it was assumed that collagen fibres originated from the cells whereas the experiments reported could be interpreted as [illegible] the intervention of cells [illegible]

ultimately incorporated into the collagen fibrils. On the other hand, it is the polypeptide chains which are in solution and are precipitated by the [illegible] saccharides or by other methods

[illegible] and Mlle [illegible] points of [illegible] ctly into [illegible] een very cautious in his interpretation. It was tempting to assume that because two

phenomena take place simultaneously in tissues they must be causally related. This is not necessarily the case, and it may well be that the mucopolysaccharides in the tissues have something to do with other [illegible]

[illegible]

other forces and, depending on circumstances, the solubility of the mixture might be greater or less than that of either of the components. This could not be predicted at present

DR CONSDEN asked how it was possible to determine the combinations of acid-soluble collagen with polysaccharides at higher pH values, since acid-soluble collagen is precipitated above pH 7

Dr. BAZIN answered that the existence of a combination between collagen (in solution at pH 4 3) and a polysaccharide (in solution at a pH higher than 5) is shown by the characteristic quality of the precipitate obtained

In conclusion, DR DELAUNAY agreed with all speakers and in particular with Dr. Gross and Dr Neuberger He had presented facts which are easily reproducible and submitted them to the meeting. As to the interpretation of these facts, it was another matter and would be the task of tomorrow.

MICRO-ANATOMY AND REACTIONS TO INJURY OF VASCULAR ELASTIC MEMBRANES AND ASSOCIATED POLYSACCHARIDES

THEODORE GILLMAN, MICHAEL HATHORN AND JACK PENN

During the last six years the investigations in this laboratory have been directed towards understanding the histogenesis and histochemistry of repair. Hitherto, analyses have been made primarily [illegible]
[illegible]
[illegible] 1956a, b). *Apart from understanding the healing of cutaneous lesions and of some aspects of epithelial-connective tissue relations in the pathogenesis of skin carcinomas, it has always been anticipated that some light may be thrown on repair processes generally, irrespective of their location in the body. Thus, it is considered that an appreciation of the reactions to acute and chronic lesions in the skin may elucidate, in some measure, the basic processes involved in repair, as opposed to regeneration, by connective tissues, of lesions in the liver, arteries, myocardium, etc.*

[illegible] cutaneous injuries
[illegible] deep to the elastic
[illegible] nvariable (Gillman
[illegible], contrary to some
[illegible] th, 1944), 'normal'
elastic fibres did not reappear in the scar tissue for months or even years. Fibres, stained with accepted elastic stains, frequently appear in longstanding scars But the morphology of these fibres, their arrangement in relation to the collagen bundles in scars and their other tinctorial reactions previously reported upon (Gillman *et al.*, 1954, 1955a, b) as well as *some of the other characteristics reported* on in the literature (Lansing, 1955) indicated that these were probably *not identical with the 'normal' cutaneous elastic fibrils.* On the basis of their morphology and tinctorial reactions these elastic-like fibres were shown to be distinguishable from 'normal' elastic fibres and were named *pseudo*-elastic fibres. Such pseudo-elastic fibres

(P.E.F.s) were shown to be the main components of senile elastosis and the so-called increase in elastic tissue or 'basophilic collagen' regularly encountered and commonly described in the skin, in association with chronic radiation dermatitis in man. The experimental production, in man *in vivo*, of localized patches of such pseudo-elastic fibre formation in the dermis has also been reported upon from this laboratory (Gillman *et al.*, 1953; 1955b).

In attempting to characterize them, these fibres were compared with the thick wavy elastic laminae or membranes in the aorta, [illegible] injury.

The objects of the present report are to outline briefly firstly, some of the morphological and tinctorial reactions of vascular elastic membranes (V.E.M.s) in healthy arteries and secondly, the changes which these undergo following injury in man, and especially (in different parts of the arterial tree) in rats given toxic doses of calciferol. Some of the implications of these findings will also be reviewed.

We are painfully aware of the limitations of staining methods in defining chemical changes in tissues, but nevertheless we firmly believe that careful observations, such as those recorded here, often form the starting-point for investigations which will ultimately lead to clarification of fibre structure, by the use of the most modern histochemical, chemical and biophysical methods and criteria, most of which are not available to our group. Some indication of what may be achieved if attempts are made to explain the tinctorial reactions, used widely, effectively, and for many years by histologists and histopathologists, is available from a recent outstanding publication by Glegg, Eidinger and Leblond (1953). By the combined use of histological, chemical and physical methods, these investigators were able to provide a satisfactory chemical explanation for some of the differences in staining properties of reticular and collagenous fibres.

We sincerely hope that the precise nature of the tinctorially definable alterations in connective tissue fibres in dermis, blood

vessels and gall bladder, outlined previously and below, will also soon be elucidated in terms of the chemistry and ultrastructure of connective tissue fibres.

MATERIAL AND METHODS

Aortae, carotids, pulmonary and other medium and small arteries and arterioles were obtained, at post mortem, from human subjects immediately after acute traumatic death (10–30 minutes) or dying from various diseases. Serial wax sections of these vessels, fixed in formalin, were stained by the methods previously outlined (Gillman *et al.*, 1953; 1955a, b; Gillman and Penn, 1956a, b) to demonstrate cytology and elastic, collagen and reticulin fibres by the periodic-acid Schiff (P.A.S.) and von Kossa (V.K.) routines – to demonstrate polysaccharides and mineralization respectively – and by the alcoholic toluidine blue method recommended by Glick (1949) for identifying metachromatic mucopolysaccharides in connective tissue ground substance and in mast cells.

The experimental material comprised heart and coronary vessels, aorta (4 parts – i.e. ascending, thoracic, pre-renal abdominal and post-renal abdominal), spleen, stomach, intestine and other tissues [illegible]

[illegible] moribund animals or rats sacrificed between the fourth and the forty-third day after the initiation of the experiments. Tissues from these animals were prepared in the same ways as outlined above for human materials.

OBSERVATIONS

1. *Morphology and Histochemistry of Normal Elastic Membranes*

Some of the staining reactions of vascular elastic membranes (V.E.M.s) have been recorded previously (Gillman *et al.*, 1955a). In particular it has been shown that these membranes seem to be composed of axial homogeneous 'cores' having tinctorial reactions different from the material which seems to constitute a thin, surrounding coating in larger vessels or an enmeshing spiral of fibrils in smaller vessels (see below). Only some of the staining qualities of these 'cores' are the same as those of the fine elastic fibrils encount-

ered in the dermal stratum papillaris and in the other loose areolar connective tissues.

Distinct differences in staining between the axial cores of the V.E.M.s and fine, elastic fibrils have been demonstrated and previously enumerated (Gillman *et al.*, 1955a, b). Moreover, in reticulin preparations of human arteries, a fine and almost continuous sheath of reticulin fibres is regularly encountered, extending along the outer borders of the wavy cores of the V.E.M s (Fig. 1). From these outer reticulin sheaths, numerous fine reticulin fibrils extend into the surrounding interstitial collagen and unite with the peri-muscular reticulin network. In Wilder preparations, counterstained with Van Gieson's stain, the 'core' of the V.E.M.s, lying within the reticulin 'sleeve', stains yellow, while the neighbouring [illegible]

ticulin and collagen meshwork (see legend and lower portion of Fig. 19 at *a*).

Polysaccharides (as demonstrated with P.A.S.) form a distinct [illegible]

is irregularly distributed in the intermembranous collagen, but is distinctly aggregated as a coating around the pale blue-staining V.E.M.s. In Rinehart-Abul Haj preparations (a modified Hale's ferrocyanide method) the deep blue-staining mucopolysaccharides are less widely distributed and regularly form a coating around the yellow-staining membranes. In the normal rat's aorta the peri-membranous reticulin sleeve is finer and more granular than that in man (Fig. 2), the P.A.S. positive sleeve around the V.E.M s is distinct although thin, but only minimal amounts of metachromatic material are detectable, scattered among the inter-membranous muscle bundles, and forming an irregular interrupted peri-membranous sheath.

Staining with Mallory's Phosphotungstic Acid Haematoxylin (Mallory's P.A.H.) demonstrates, in human aortic V E M.s, a positively staining membrane with occasional 'moth-eaten' clear spaces along its length. This blue-stained sleeve around the wavy membrane is closely hugged by the surrounding orange-staining collagen. In the human aorta this sleeve around the elastic mem-

branes (which stains deep blue to purple with Mallory's P.A.H.), is virtually unbroken. However, further insight into their possible structure is obtained from the study of smaller arteries in man and of the aorta in the rat. In the internal *elastic membranes of the small* visceral arterioles in man (Fig 3) the material, staining blue with [illegible]
[illegible]
[illegible]
[illegible]
[illegible]
facts. Firstly on focusing up and *down, on such internal elastic* membranes, the blue 'dots' seem to join one another, giving the [illegible]

blue stripes (Fig. 4*b*). This impression, gained from the study of medium-sized arteries, is reinforced by the findings in terminal arterioles In the latter, the collagen-like core seems thicker and more homogeneous, while the surrounding spiral of blue-staining fibrils seems to be less tightly coiled In sections, stained with elastic stains (orcein, Weigert's resorcin-fuchsin or Verhoeff's methods), *the positively stained material is coarsely granular (Fig. 5), a non*-staining structure being occasionally visible between these coarse granules. However, with Mallory's P.A.H. (Fig. 6 – a serial section of the same arteriole shown in Fig. 5), the collagen-like portion of the internal elastic membrane is prominent, being flanked by rows of very *fine blue-staining granules.*

Thus, it would seem, that in man, the collagen-like core of the elastic membranes, in larger arteries, is surrounded by an almost continuous, fairly thick coat of some other material (perhaps elastin-like or reticulin-like) while, as the arteries become smaller, so the collagen-like core becomes thicker and is surrounded by a thinner coating of this other material, which now forms a spiral of blue-staining fibrils – this spiral becoming progressively more loosely coiled and constituted of finer fibrils as one proceeds to the smallest branches of the arterial tree.

These findings and interpretations, concerning the arterial elastic membranes in man, are further confirmed from a study of the arter*ies in rats. In the normal aorta of the rat,* the V.E.M.s are clearly

constituted of a core of material staining orange with Mallory's P.A.H. These cores are surrounded and connected with one another by fine blue-staining fibrils (Fig. 18) having a disposition different from reticulin (compare Figs. 2, 18 and 19). Following injury, the 'cores' become thicker and the inter- and peri-membranous blue- [illegible] (compare the [illegible] preparations of [illegible] ed pale brown while the outer portions are heavily stained. These differences in staining are particularly clearly visible in oblique sections of these V.E.M.s, in which deep brown stripes can be seen spiralling around the paler staining cores.

Thus, from the histological evidence here outlined, it seems that the V.E.M.s in arteries: (1) Are not homogeneous in structure, being constituted of a core of collagen-like material, sleeved by polysaccharide and possibly some other fibrillar element similar to elastin. (2) Differ tinctorially, morphologically and histochemically from elastic fibrils found in skin and loose areolar connective tissue. (3) Are structurally closely inter-related with the surrounding reticulin, collagen and polysaccharides. (4) Structures delineated with elastic stains, and apparently homologous in the larger arteries of the rat and man, differ morphologically and tinctorially when other criteria are used.

These morphological and tinctorial findings in healthy arteries are substantiated by and permit a fuller understanding of the reactions to injury.

2 *Reactions of Elastic Membranes to Injury*

In man, damage of elastic membranes is invariably followed by reactions indistinguishable from those seen in healing cutaneous injuries, i.e. fibroblast, giant cell, mucopolysaccharide and collagen accumulation (Fig. 7) with subsequent scar formation. These are the reactions of repair of injury as opposed to regeneration. Reactions, similar to th[illegible] [illegible] described recently in

[illegible]

strated, in recent months, that the widespread lysis of vascular elastic membranes, induced by feeding sweet-pea seed flour to rats, is invariably followed by extensive fibroblastic reactions with scar

branes (which stains deep blue to purple with Mallory's P.A.H.), is virtually unbroken. However, further insight into their possible structure is obtained from the study of smaller arteries in man and of the aorta in the rat. In the internal elastic membranes of the small visceral arterioles in man (Fig. 3) the material, staining blue with [illegible]

facts. Firstly *on focusing* up and down, on such internal elastic membranes, the blue 'dots' seem to join one another, giving the optical impression of *spiralling, an impression not transmissible in* stills taken at one plane of focus. Secondly, in oblique sections of such membranes (Fig. 4) the clear homogeneous collagen-like orange-stained core is either flanked by blue fibrils (Fig. 4*a*) or has distinct blue stripes (Fig. 4*b*). This impression, gained from the study of medium-sized arteries, is reinforced by the findings in terminal arterioles. In the latter, the collagen-like core seems thicker and more homogeneous, while the surrounding spiral of blue-staining fibrils seems to be less tightly coiled. In sections, stained with elastic stains (orcein, Weigert's resorcin-fuchsin or Verhoeff's methods), the positively stained material is coarsely granular (Fig. 5), a non-*staining structure being occasionally visible between these coarse* granules. However, with Mallory's P.A.H. (Fig. 6 – a serial section *of the same arteriole shown in Fig. 5*), the collagen-like portion of the internal elastic membrane is prominent, being flanked by rows of very fine blue-staining granules.

Thus, it would seem, that in man, the collagen-like core of the elastic membranes, in larger arteries, is surrounded by an almost continuous, fairly thick coat of some other material (perhaps elastin-like or reticulin-like) while, as the arteries become smaller, so the collagen-like core becomes thicker and is surrounded by a thinner coating of this other material, which now forms a spiral of *blue-staining fibrils – this spiral becoming progressively more* loosely coiled and constituted of finer fibrils as one proceeds to the smallest branches of the arterial tree.

These findings and interpretations, concerning the arterial elastic membranes in man, are further confirmed from a study of the arteries in rats. In the normal aorta of the rat, the V.E.M.s are clearly

constituted of a core of material staining orange with Mallory's P.A.H. These cores are surrounded and connected with one another by fine blue-staining fibrils (Fig. 18) having a disposition different from reticulin (compare Figs. 2, 18 and 19). Following injury, the 'cores' become thicker and the inter- and peri-membranous blue-staining fibrils seem to disappear in a patchy fashion (compare the upper and lower parts of Fig. 18). In orcein-stained preparations of the normal rat's aorta, the cores of the V.E.M.s are tinted pale brown while the outer portions are heavily stained. These differences in staining are particularly clearly visible in oblique sections of these V.E.M.s, in which deep brown stripes can be seen spiralling around the paler staining cores.

Thus, from the histological evidence here outlined, it seems that the V.E.M.s in arteries. (1) Are not homogeneous in structure, being constituted of a core of collagen-like material, sleeved by polysaccharide and possibly some other fibrillar element similar to elastin. (2) Differ tinctorially, morphologically and histochemically from elastic fibrils found in skin and loose areolar connective tissue. (3) Are structurally closely inter-related with the surrounding reticulin, collagen and polysaccharides. (4) Structures delineated with elastic stains, and apparently homologous in the larger arteries of the rat and man, differ morphologically and tinctorially when other criteria are used.

These morphological and tinctorial findings in healthy arteries are substantiated by and permit a fuller understanding of the reactions to injury.

2. *Reactions of Elastic Membranes to Injury*

In man, damage of elastic membranes is invariably followed by reactions indistinguishable from those seen in healing cutaneous

similar to those here portrayed, have also been described recently in the carotid artery 'elastic granuloma' in man. The damaged elastic membranes seem to act as foreign bodies evoking giant cell formation (Fig. 8). Walker and Wirtschafter (1956) have also demon-

formation. Unfortunately, the latter authors made no mention of the reactions of the elastic fibrils in skin, lungs, elastic cartilages and areolar connective tissues.

In man, damage of the media of smaller arteries, as previously described (Gillman *et al.*, 1955a), may be associated with the

ally demonstrable changes are regularly encountered. Mineralization (von Kossa positive material) of the myocardium and of the intimal portions of the coronary arteries (Fig. 9) ensues within 4-6 days of the first dose of calciferol. These cardiac lesions usually resolve rapidly so that by the tenth day von Kossa positive material is no longer detectable either in the myocardium or in the coronary arteries. The former now show [illegible]
infiltrations (with or without
coronaries now present a marke
line) strongly P.A.S. positive int
and twelfth days the internal pa
(Fig. 15), then of the thoracic and later of the abdominal aorta becomes heavily mineralized, as may also the alveolar portions of the lungs and usually, in addition, parts of the gastric mucosa, the musculature of the stomach and of the colon. The mineralization of the alimentary tract is usually most marked between the eleventh and eighteenth days of the experiment. After this time, most (but not all) of the von Kossa positive material disappears from the various parts of the aorta, and from the alimentary tract, so that, by the fortieth day (i.e. 35 days after the last dose of calciferol) only isolated sections of the ascending and thoracic aorta retain von Kossa positive material (Fig. 20). The cardiac muscle regenerates completely, while the coronary arteries and the severely damaged portions of the aorta undergo varying degrees of fibrosis.

Details of these reactions, which are similar to those described by other authors (Sohl *et al.*, 1930; Ham and Portuondo, 1933; Ham and Lewis, 1934) have been presented elsewhere (Gillman *et al.*, 1956). Pertinent to the present discussion are the changes in the coronary arteries, aorta and smaller vessels – the latter not having been mineralized at any stage of the experiments.

At the outset it must be stated that in *all* loci, accumulations of von Kossa positive material are consistently associated with marked

increases in P.A.S. positive material (compare Figs. 9 and 10). ... material (apart from that in the

... of alcohol-

... ucopolysac-

charides (M.P.S.) with or without fibrosis (Figs. 20 and 21) – depending on the intensity and duration of the damage.

On superficial examination, the von Kossa positive material seems to be deposited within the elastic membranes. However, careful study of the reactions preceding, during and succeeding mineralization reveals that the calcium (and other von Kossa positive mineral salts) is not deposited within the elastic membranes, but rather around them. This is clearly demonstrated for the aortic elastic ...

tween the previously mineralized V.E.M.s (Figs. 20 and 21). Apparently, only if the mineralization is very severe or prolonged (as occurs especially in the first part of the aorta of rats, fed larger doses of calciferol) do the V.E.M.s themselves become disrupted, leading to irregularities in their arrangement in the vascular walls, very marked mucopolysaccharide accumulations, frequently associated with ulceration of the intima, and subsequent giant cell and fibroblastic reactions (Figs. 20 and 21).

Coronary arteries, which mineralize early and then very rapidly lose such excessive minerals, can easily be distinguished from the normal coronary arteries, even by the tenth day of the experiments (Fig. 10). By this time, when von Kossa positive material has usually disappeared from the ...

... or twelfth day the internal elastic membranes (normally prominent) have disappeared from previously mineralized coronary arteries. By the forty-third day, the walls of coronary arteries have thickened considerably, and the lumina have been narrowed as a result of the replacement of the walls by fibroblasts and the marked accumulation of P.A.S. positive and metachromatic polysaccharides (Figs. 13 and 14). Similar changes are observable, although in lesser degree, in medium-sized peripheral arteries, which do not seem to become mineralized even during the early phases of the experiments.

Some of the histologically demonstrable reactions of the aorta (to such severe but transient intoxication with calciferol) are depicted in Figs. 15 to 21. The extent of initial calcification of the ascending aorta is indicated in Fig 15. In Figs. 16, 16*a* and 17 the disposition of von Kossa positive material in the thoracic and ascending aorta, respectively, are shown, 5 days after the last dose of calciferol. It can here be seen that mineralization occurs predominantly between and around the elastic membranes, the cores of the latter being visible as clear stripes within the black-staining mineral material (Fig. 16). Figs. 16 and 16*a* – high power views of serial sections of the calcified elastic membranes in the thoracic aorta – clearly reveal firstly, the intactness of the membranes (Fig. 16*a*) which are heavily coated with von Kossa positive material in Fig. 16; secondly, in Fig. 16*a*, the dark-staining metachromatic M.P.S.s accumulated around these intact, although calcified, membranes.

Serial sections of the ascending aorta (seen at low power in Fig. 15) are presented to demonstrate the following:

[illegible] and inter-[illegible], as opposed [illegible] and 19). [illegible] res and the [illegible] ing fibrillar material around these swollen V.E.M.s (compare the lower part of Fig. 18 – 'normal cores' – with the middle portion – 'swollen cores' – in this specimen stained with Mallory's P.A.H.).

[illegible]

figure.

[illegible] residual patchy mineral- [illegible] the media and the overlying intima is undergoing fibrosis – i.e. [illegible]

[illegible] cutaneous wound.

Thus, the morphological and tinctorial findings, in healthy blood vessels, showing that the V.E.M.s are not homogeneous in structure, are supported by the reactions induced in these membranes by injurious agents.

COMMENTS

It is possible to demonstrate that the vascular elastic membranes (V.E.M s) in arteries are morphologically, tinctorially and histochemically distinguishable from the fine elastic fibrils in areolar connective tissue, pulmonary alveoli and elastic cartilages. It would *seem that the 'core' of these V.E.M.s is maintained by a specialized* alteration of the connective tissue components immediately related to these membranes. Reactions to injury, exemplified in rats by the metastatic mineralization following toxic doses of calciferol, indicate that the site of predilection for such mineralization is *not* the core of the V.E.M.s but rather their encasing reticulin and polysaccharide 'sleeves'. This sleeving of elastic membranes by polysaccharides and even by recognizable fibrils, here described, is in conformity with similar ground substance sleeves around corneal fibres described by van den Hooff (1952). The membranes themselves do not seem to be calcifiable, and in this respect resemble other collagen fibre bundles. On the other hand, the reticulin and polysaccharides coating the V.E.M.s, like similarly composed ground substance of bones and cartilages, seem especially liable to mineralization.

Taken together, the morphological and histophysiological evidence here presented, suggests that the integrity of the V E.M s is a function of the continuous activity of the surrounding connective tissue components, directed towards the maintenance of the membranes by the accretion and removal of molecules at their surfaces. This allows for constant regeneration of wear and tear trauma. The membranes themselves seem to act as 'organizers' for the metabolic activity of the surrounding polysaccharides and other connective tissue components, for, once these membranes are disrupted or destroyed, the connective tissues do not seem capable of replacing them easily. Such severe or prolonged injuries to the peri-membranous connective tissue are consequently succeeded by the usual reactions to injury, such as those seen in other parts of the body (e g. in the skin), *and characterized by M P.S* accumulation,

of those activities of the vascular connective tissues norm cerned in maintaining the integrity of the V.E.M.s. Suc progress through vascular *repair* (as opposed to regenera culminate in fibrosis – the vascular equivalent of cutaneous

(4) As in the repair of all injuries to connective tissu enough to invoke repair, so too in vessels, one of the characterized by that accumulation of mucopolysaccharides which regularly precedes fibrosis.

(5) Mineralization, lipoidal changes or the development of pseudo-elastic fibres seem to be determined primarily severity, duration and nature of the disturbances in the pe branous connective tissue polysaccharide metabolism induc injury. This is indicated by the finding that experimentally mineralization of the V.E.M.s in rats first occurs *around* within these membranes. Only when the latter are disrup fibrosis, or the other changes mentioned above, superve V.E.M.s thus seem to act, in some measure, as organizers of tive tissue metabolism in arteries.

(6) It is suggested that the varying susceptibility to i different parts of the vascular tree may be a function firstl different rates of polysaccharide turnover in the peri-m connective tissues, secondly of the nature and severity of th and thirdly of the prevailing metabolism.

(7) Most of the reactions to injury, observable in blood *can* be interpreted in terms of the usual histogenesis of the r injuries to connective tissues, no matter where they occu body. Such interpretations may be facilitated by the finer anatomical details here outlined.

ACKNOWLEDGMENTS

The authors are glad to acknowledge the very generou *from the Schlesinger Organization* which made the executi presentation of this work possible.

... for her grant

... his Bil

... studies

... photo

graphs. Mrs. B. H. Robinow has at all times been most


10
11
12
13
14
15
16
16a
17
18
19

PLATE II

FIG 9

Heart and coronary arteries of a rat, one day after the last of five daily doses of 36,000 units of calciferol (i e sixth day of experiment) showing von Kossa positive walls of coronary vessels. Von Kossa preparation × 50

FIG 10

Same field as shown in Fig 9 but several sections away showing intense P A S positive staining of entire walls of medium-sized coronary vessels Periodic-Acid Schiff (P A S) preparation × 50

FIG 11

Normal medium-sized coronary artery of a rat showing wavy, indented P A S positive internal elastic membrane P A S stain × 220

FIG 12

Small coronary vessel in a rat 5 days after the last of five consecutive daily doses of 36,000 units of calciferol to demonstrate intense broad P A S positive, internal elastic membrane and deposition of P A S positive material above and around the vessel There is also an increased peri-vascular cellularity indicative of incipient fibrosis P A S method × 220

FIG 13

Markedly thickened cellular, fibrotic medium-sized coronary vessel in the heart of a rat 40 days after the last dose of calciferol Note also the red-staining pools of P A S positive material (grey in figure) towards 6 o clock and 2 o clock on this vessel P A S method × 220

FIG 14

Serial section to vessel shown in Fig 13 demonstrating again marked fibrosis and pools of metachromatic mucopolysaccharide (especially well shown at 6 o'clock and between 12 and 2 o'clock on the walls of this vessel) Alcoholic toluidine blue only × 220

FIG 15

Extent of mineralization, confined to the intimal portion of the media of the ascending aorta in a rat one day after the last of five daily doses of 36,000 units of calciferol Von Kossa method × 23

FIG 16

to demon-
The central
von Kossa

FIG 16a

Serial section to Fig 16 to show intact elastic membranes surrounded by darkly staining metachromatic polysaccharide which is also aggregated in pools around the uppermost elastic membrane in this figure Alcoholic toluidine blue only × 500

FIG 17

Appearances of von Kossa preparation (same ascending aorta as shown in Fig 15) to show distribution of von Kossa positive material in relation to wavy elastic membranes towards the inner aspect of the media Von Kossa method × 500

FIG 18

Serial section to Fig 17 to show thickened cores of vascular elastic membranes towards intima surrounded by pools or streaks of reticulin or elastin-like material compare with healthy membranes and intermembranous fibrils below Mallory's P A H × 500

FIG 19

Reticulin method applied to serial section to Fig 18 demonstrating the thickening of the cores of the elastic membranes (towards top half of picture) and granular reticulin sheaths around these inner damaged membranes The spiral nature of the reticulin fibres is also clearly shown around the apparently undamaged elastic membranes of the outer media (at a) Wilder reticulin preparation × 500



PLATE III

Fig 20

[illegible]

area of residual calcification with a plaque projecting towards the intima Von Kossa preparation × 150

Fig 21

[illegible]

and helpful in obtaining journals in very difficult circumstances. Finally, it is a pleasure to express our sincere thanks to Mrs. L. Wisselo for her efficient and patient secretarial assistance throughout these investigations.

We are also indebted to Dr. A. E. Gremeaux of Roussel Laboratories Ltd. (London) for a supply of purified calciferol, Dr. R. L. Craig of G. D. Searle & Co. for a supply of sulphated hyaluronic acid, and to Dr. G. H. Berryman of Abbott Laboratories for a supply of heparin, used in related experiments.

GROUP DISCUSSION

Dr Robb-Smith could not agree with Dr. Gillman that elastic tissue in [illegible] though the pattern is never quite identical with the original subepidermal network. However, if there has been marked loss of tissue or inco-ordination between the epithelial and connective tissue regeneration as a result of infection, foreign bodies, etc, then elastic tissue regeneration was irregular or non-existent He had confirmed these findings in experiments on rabbits, and there was no doubt that regeneration did occur although it was difficult to obtain and occurred slowly. He found Dr Gillman's pseudo-elastic fibres of very great interest and agreed that they had not [illegible]

salts, he recalled that elastic tissue was one of the first tissues to take up injected colloidal solutions, for example trypan blue. This dye was later removed to other tissues, particularly the reticulo-endothelial system. Vitamin E deficiency in animals will reproduce similar aortic changes very nicely. The only point at issue with Dr. Gillman was the question of regeneration of elastic tissue.

Dr. Gillman said that elastic tissue was not easily regenerated, at least not in scars He had examined a wide range and number of scars in man and animals, the scar tissue varying in age from 2 days to 20 years, and even in very old scar tissue the elastic fibres which did reappear were abnormal, that is to say that, although elastic-like fibres reappear in *old* scars, they did not have a 'normal' morphology and did not acquire the original 'normal' pattern of arrangement. *In vivo*, trypan blue staining of

aorta seems to be due to dye accumulation in the 'sleeve' round the core of the aortic elastic membranes. In odoratism (sweet pea seed poisoning) fibrosis occurs only after rupture of the elastic membranes; this again indicating that damage of the elastic component may be an essential precursor of repair.

DR. BALÓ recalled the various forms of elastic regeneration as exempli- [illegible]

[illegible] a misinterpretation by histologists In reality, they were examining an accumulation of *pseudo*-elastic fibres, as defined by the methods and criteria outlined, rather than of new 'normal' elastic fibres. He wanted to stress the fact that calcification occurred not *in* but *around* the elastic membranes and can appear and disappear without fibrosis necessarily following. Large doses of calciferol which lead to severe or prolonged calcification followed by disruption of the elastic laminae were usually succeeded by foreign body reactions and fibrosis, in other words, repair, [illegible]

[illegible] elastin.

DR GILLMAN said no, to his knowledge there was no reconstruction of the subepidermal elastic network of the skin in such striae. The depth of the injury seems to determine the type of subsequent reaction. Surface abrasions of epidermis were followed by complete regeneration of original architecture whereas wounds extending deep to the subepidermal elastic network always appeared to produce scar formation.

[illegible] elastase [illegible] cium [illegible] the [illegible] the [illegible] ally [illegible] tion must be bound to the tissue itself. *It is quite conceivable that the* pre-calcification triggers off the medial degeneration in arteriosclerosis.

DR. GILLMAN pointed out that after injury, several minerals including iron, seem to bind on to the peri-elastic 'sleeve' he had described. Such depositions do not occur in normal 'sleeves', no matter how high the blood calcium, but readily occur after injury.

DR REED stated that several of Dr. Gillman's findings in aortic elastin were confirmed at the electron microscopic level There were two different structural forms of aortic elastin· fibres and sheets. There was also a

close association with reticulin and collagen fibrils, whilst the elastic fibres were coated with dense amorphous material which probably represented mucopolysaccharides or a mucoprotein system. Had Dr. Gillman made any observations on local tissue pH values in the areas damaged by burns, ultraviolet light, etc.?

DR. GILLMAN replied, no, he had not

DR HALL stated that studies of dermal preparations from senile elastosis showed that the masses of material which appeared to be elastic fibres when stained and examined under the light microscope, proved to be degraded collagen when examined under the electron microscope. The pseudo-elastic fibres demonstrated by Dr. Gillman might similarly represent the products of a reaction stopped part way in the process of conversion of collagen to elastin. He believed that normally, collagen was degraded and then rebuilt to form elastin, but that under certain conditions, either through the lack of some intermediate or enzyme, the process was stopped half way. The conversion might be considered as having proceeded far enough to produce the tinctorial properties of elastic fibres, but not far enough to produce their physical properties.

DR SYLVÉN called attention to the high mass content of the elastic membranes, revealed by microradiography, as compared to the low mass of the surrounding connective tissue of the vascular walls. The mass distribution as such seemed to have bearings on the non-specific staining reactions described by Dr Gillman, and might partly explain the microscopical appearance of the suggested 'core' structure

DR GILLMAN stated that it would appear that the 'core' of these pseudo-elastic fibres possessed staining properties similar to those of collagen There seemed to be a relation between four things in the skin and arteries, interfibrillar and intercellular substance, reticulin, collagen and elastic fibres Disturbances of fibrillogenesis seemed to lead to the production of the abnormal structures which he had described

Finally, DR GROSS showed one lantern slide of fibres obtained from marine sponges This demonstrated that two differently staining fibres could both be collagen as defined by X-ray diffraction, electron microscopy and chemical composition

DR. GILLMAN responded that this did not prove anything other than that Dr Gross had yet another problem on his hands, namely to account for differences in arrangement of apparently identical electron microscopic fibrils, inter- and peri-fibrillar components responsible for the differences in staining reactions and morphology seen with the histologists' techniques This would permit satisfactory progress from the presently known to the unknown.

Another possible explanation was that the fibroblasts might secrete the material under question. However, it would seem reasonable to expect some histochemical indication of such a secretion before the appearance of the metachromatic component, e.g. in the shape of some granular or other strongly metachromatic fibroblast material, and further to find an increasing concentration gradient around the first foci of growing fibroblasts. Such observations have, however, not been made, and therefore we are still inclined to assume that the mast cells may be implicated in this phenomenon.

To test this hypothesis, we planned to extract granulation tissue as a first step by means of salt solutions including histological control of tissue residues. Since the material was mainly located in the ground substance proper, it would seem possible to get it in solution before other polysaccharide material more closely bound to structural proteins. The KSCN extraction method was originally instigated for the selective extraction of the native heparin complex from the tissue mast cells (Julén, Snellman and Sylvén, 1950).

In other words, it was thought possible that the tissue mast ce might contribute to the polysaccharide components of the groun substance during repair. Thus did not mean that the mast cel would 'secrete the ground substance' as such, since it is realized tha ground substance components are probably derived from severa sources (cf. review by Sylvén, 1956).

MATERIALS AND METHODS

The control material embodies strips of pooled abdominal wall from adult albino rats, including the skin together with muscle and extraperitoneal fat tissue. Mast cells occur mainly in the skin proper, and no or very little metachromatic ground substance is found in this material. In the test series similar rats were subjected to laparatomy under sterile conditions, the mid-line incisions extending from the thorax nearly to the symphysis. On the third day after the operations, when the largest amounts of metachromatic ground substance and growing fibroblasts were expected, the healing wounds were cut out with scissors. The granulation tissue as such could not easily be isolated and consequently part of the surrounding skin and muscle had to be included. Infected cases were excluded. Pooled materials were kept frozen in solid CO_2 until further use.

RESULTS

The material precipitated at 70 per cent ethanol was determined. At such high alcohol concentrations some salts will also precipitate. The total yields obtained are therefore not very reliable. The yields of heparin in Table I have been calculated from the anti-clotting

TABLE I

	Normal Rat Skin		*Granulation Tissue, 3 days old*	
Experiment No.	*Total yield* in mg/kg w w*	*Amount of Heparin in mg/kg w w*	*Total yield* in mg/kg w w*	*Amount of Heparin in mg/kg w w*
1	257*	23 4	184*	7 7
2	285	54 3	146	5 6
3	146	47 3	160	1 8
4	149	22 9	240	1 2
5	49*	23 5	53	1 2
6	—	—	167*	10 0
	Average yields	35 mg/kg w w		47 mg/kg w w

* Including varying amounts of co-precipitated salts.

assays. All figures given in the table are calculated on a wet weight basis.

This did not seem very promising. Upon further consideration, however, the suggestion arose that some new material, appearing in

Following the addition of ethanol up to a concentration of 50 per cent (vol), a Fraction A was obtained, which contained all the metachromatic and anti-clotting material. Another Fraction B was precipitated by an ethanol concentration between 50 and 60 per cent. This substance was non-metachromatic and devoid of anti-clotting activity. The heparin content of Fraction A, determined by the anti-clotting assay, was as follows:

FRACTION A	from normal rat skin	35 mg. heparin per kg.w.w.
FRACTION A	from 3-days-old granulation tissue	36 mg. heparin per kg.w.w.

Hydrolysis of fraction A and subsequent paper electrophoresis in borate b [illegible] glucosamin [illegible] of starting [illegible] referred to glucuronic acid and galactosamine. No protein residues were found.

Further, some anti-clotting experiments conclusively showed (1) that purified heparin (pharmaceutical) + substance 'B' (mentioned above) derived from normal rat skin, did not interfere with the anti-clotting activity of the heparin; but (2) that purified heparin + substance 'B' derived from the granulation tissue strongly inhibited the anti-clotting activity of the heparin. The nature of this peculiar material is still unknown. Fraction B contained so far only carbohydrates, and no trace of protein was detected by the biuret and ninhydrin reactions.

DISCUSSION

The results suggest that there is probably no difference in the total heparin content between the two tissue materials in question Alkaline extractions performed with the more drastic method of Charles and Scott and of Wilander yielded similar amounts of heparin in both tissues as found by means of the KSCN extraction method. No new metachromatic substance was detected. It seems therefore that the previous assumption as to the occurrence of heparin in the intercellular medium of granulation tissue will obtain additional support. This will again call attention to the possible biological significance of heparin as a component of ground substance during fibroblast proliferation and perhaps also during the subsequent fibrogenesis. The mast cell polysaccharide thus released would be free to react with basic proteins and might influence a number of reactions in the growing cells. It could act as a sulphur transfer mole- [illegible]

In the granulation tissue, the KSCN extraction [illegible] an unknown carbohydrate inhibitor of the anti-clotting effect of [illegible]

GROUP DISCUSSION

Dr Meyer pointed out that an anti-clotting factor present in granulation tissue had recently been reported in the *Federation Proceedings*, 1956. He asked if sulphated polysaccharides other than heparin had been extracted from healing wound tissue.

Dr. Sylvén replied that other acid polysaccharides, such as hyaluronic acid and chondroitin sulphate, no doubt were present, probably in increased amounts, but had so far not been extracted

Dr Meyer said in his own work, on normal rat skin injected with ^{35}S sulphate there were two types of CSA, namely, B and C in comparable concentrations to that found in pig skin The B has a negative rotation and contains chondrosamine but only the C fraction is hydrolysed by testicular hyaluronidase

Dr Sylvén said they had until now used the thiocyanate method only [illegible]

in this experiment

Dr Sylvén answered that pooled albino rat skins from animals of the same age group, 2 to 3 months old and of the same weight, about 200 gm., were utilized, more than 1000 rats being consumed for this investigation.

Dr. D S. Jackson said that on the evidence presented in his paper he would like to offer an alternative explanation of Dr Sylvén's findings. By the methods used by Slack (*in press*) the presence of heparin was not noted, whereas there was a high concentration of other sulphated polysaccharides into which $^{35}SO_4$ was rapidly incorporated even in the early stages of development. The release of pre-existing heparin by the mast cell observed by Dr Sylvén could be due to the trauma of incision. Possibly the true ground substance is formed later and hence the large uptake of $^{35}SO_4$ He emphasized that Dr. Sylvén's work was on wound healing in which a different mechanism may occur from that appertaining to the carageenin granuloma

Dr Sylvén referred to the text and the diagram given in a previous paper of his (1941) and felt this explanation was surely refuted on the grounds of the sharp decrease in the number of tissue mast cells 24-36 hours after wound infliction and the most marked and coincident rise in metachromatic ground substance material in the identical tissue region.

Dr D S Jackson said that this could still be an effect of the incision and wondered what was the explanation of the large uptake of sulphate into sulphated polysaccharide that occurred in the early stages of granulation tissue formation, since the heparin must presumably be already present in the mast cell before the wound was made

...OGY AND DIFFERENTIATION OF THE CONNECTIVE TISSUE FIBRES

WILLY SCHWARZ

A connective tissue fibre, as identified under the ordinary microscope, is heterogeneous under the electron microscope. It consists of fibrils, which are generally invisible in the ordinary microscope, and of a cementing substance which occupies the spaces between the fibrils. It is, therefore, necessary to distinguish clearly between the expressions 'fibre' and 'fibril'. The fibrils are the structural el... of a fibre. Thus, the expression 'fibril' may be made more p... for histologists generally understand by fibril, a structure lying ... threshold of visibility in the ordinary microscope, and canno... whether it is a fibril in the sense of an element or merely a thin ... including both fibrils and the cementing substance. In histol... based on the ordinary microscope one recognizes besides ela... fibres, two other types, namely, argyrophil and collagenous fib... As electron microscopy reveals, both of the latter fibre-types ha... fibrils which can be characterized by the well-known comple... periodic cross-striations. It has been suggested on the basis of thi... finding that histological differences between these fibre-types are conditioned, not by differences in their fibril-elements, but by the varying constitution of their cementing substances (Wassermann, 1956). For this reason, one speaks on principle of collagenous fibrils in connection with these periodic, transversely striated fibrils and regards the striation as the 'fingerprint' of collagenous fibrils (Gross, 1949, 1950). An argyrophil fibre consists accordingly of collagenous fibrils and a cementing substance typical for reticulin.

Since we assume the formation of fibrils to be basically extracellular, it is hard to imagine how *identical* fibrils are formed in different tissues and organs out of different cementing substances. Here we have to ask whether the observation of identical cross-triations is a sufficient criterion of identity of the fibrils. One ould, for instance, interpret the cross-striations merely as a structural characteristic common to all connective tissue fibrils. In that se, the cross-striations would not be the trade-mark for collagen

alone, but simply for a connective tissue fibril. When one wants to differentiate connective tissue fibre-types by electron microscopy, one must proceed with the aid of other criteria as in histology. These can operate not only for the classification of individual con-

One of these criteria is, of course, the varying mode of silvering of the connective tissue fibrils observed in the electron microscope

I should like to demonstrate the method of silvering collagenous fibrils with the example of plantar aponeurosis. Histologically this is a tendinous structure and one is dealing with collagenous fibres. When one isolates the collagenous fibrils by ultrasonic vibration, and then silvers them, most of the fibrils acquire silver particles in the region of the dark band.

The contrasting strengthening of the dark bands by the silver granules makes the transverse striation period of the fibrils appear more prominent (Fig. 1*c*). This finding was first described by Dettmer, Neckel and Ruska (1951) for tendon collagen of cattle, and then demonstrated by Schwarz (1953) for the sclera, by Probst and Ratzenhofer (1953) and Pahlke (1954) for human tendon, and by Linke (1955) for human skin. One could take the view that this involves, strictly speaking, not an implantation of silver particles in the fibrils, but rather a surface absorption of silver particles *on* the fibrils, a matter that cannot always be determined with absolute certainty when dealing with isolated fibrils. In order to clarify this question, I have also used the ultra-thin-section method (Fig. 1*b*). If the silver were deposited on the surface of the fibrils, longitudinal sections of collagenous fibrils would be free of silver unless a fibril

one must speak of internal silvering of the collagenous fibrils. Irving and Tomlin (1954) do not believe in an internal silvering of the fibrils and ascribe the surface silvering which they found around

MORPHOLOGY AND DIFFERENTIATION OF THE CONNECTIVE TISSUE FIBRES

WILLY SCHWARZ

A connective tissue fibre, as identified under the ordinary microscope, is heterogeneous under the electron microscope. It consists of fibrils, which are generally invisible in the ordinary microscope, and of a cementing substance which occupies the spaces between the fibrils. It is, therefore, necessary to distinguish clearly between the expressions 'fibre' and 'fibril'. The fibrils are the structural elements of a fibre. Thus, the expression 'fibril' may be made more precise, for histologists generally understand by fibril, a structure lying on the threshold of visibility in the ordinary microscope, and cannot say whether it is a fibril in the sense of an element or merely a thin fibre including both fibrils and the cementing substance. In histology based on the ordinary microscope one recognizes besides elastic fibres, two other types, namely, argyrophil and collagenous fibres. As electron microscopy reveals, both of the latter fibre-types have fibrils which can be characterized by the well-known complex periodic cross-striations. It has been suggested on the basis of this finding that histological differences between these fibre-types are conditioned, not by differences in their fibril-elements, but by the varying constitution of their cementing substances (Wassermann, 1956). For this reason, one speaks on principle of collagenous fibrils in connection with these periodic, transversely striated fibrils and regards the striation as the 'fingerprint' of collagenous fibrils (Gross, 1949, 1950). An argyrophil fibre consists accordingly of collagenous fibrils and a cementing substance typical for reticulin.

Since we assume the formation of fibrils to be basically extracellular, it is hard to imagine how *identical* fibrils are formed in different tissues and organs out of different cementing substances. Here we have to ask whether the observation of identical cross-striations is a sufficient criterion of identity of the fibrils. One could, for instance, interpret the cross-striations merely as a structural characteristic common to all connective tissue fibrils. In that case, the cross-striations would not be the trade-mark for collagen

alone, but simply for a connective tissue fibril When one wants to differentiate connective tissue fibre-types by electron microscopy, one must proceed with the aid of other criteria as in histology. These can operate not only for the classification of individual con- [illegible]

[illegible] the connective tissue fibrils observed in the electron microscope [illegible]

[illegible] ous and argyrophil fibres.

I should like to demonstrate the method of silvering collagenous fibrils with the example of plantar aponeurosis. Histologically this is a tendinous structure and one is dealing with collagenous fibres. When one isolates the collagenous fibrils by ultrasonic vibration, and then silvers them, most of the fibrils acquire silver particles in the region of the dark band.

The contrasting strengthening of the dark bands by the silver granules makes the transverse striation period of the fibrils appear more prominent (Fig. 1*c*). This finding was first described by Dettmer, Neckel and Ruska (1951) for tendon collagen of cattle, and then demonstrated by Schwarz (1953) for the sclera, by Probst and Ratzenhofer (1953) and Pahlke (1954) for human tendon, and by Linke (1955) for human skin. One could take the view that this involves, strictly speaking, not an implantation of silver particles in the fibrils, but rather a surface absorption of silver particles *on* the fibrils, a matter that cannot always be determined with absolute certainty when dealing with isolated fibrils. In order to clarify this question, I have also used the ultra-thin-section method (Fig. 1*b*). If the silver were deposited on the surface of the fibrils, longitudinal sections of collagenous fibrils would be free of silver unless a fibril surface lay directly at the level of sectioning The described periodic silvering of the fibril [illegible]

one must speak of internal silvering of the collagenous fibrils. Irving and Tomlin (1954) do not believe in an internal silvering of the fibrils and ascribe the surface silvering which they found around

MORPHOLOGY AND DIFFERENTIATION OF THE CONNECTIVE TISSUE FIBRES

WILLY SCHWARZ

A connective tissue fibre, as identified under the ordinary microscope, is heterogeneous under the electron microscope It consists of fibrils, which are generally invisible in the ordinary microscope, and of a cementing substance which occupies the spaces between the fibrils. It is, therefore, necessary to distinguish clearly between the expressions 'fibre' and 'fibril'. The fibrils are the structural elements of a fibre. Thus, the expression 'fibril' may be made more precise, for histologists generally understand by fibril, a structure lying on the threshold of visibility in the ordinary microscope, and cannot say whether it is a fibril in the sense of an element or merely a thin fibre including both fibrils and the cementing substance. In histology based on the ordinary microscope one recognizes besides elastic fibres, two other types, namely, argyrophil and collagenous fibres As electron microscopy reveals, both of the latter fibre-types have fibrils which can be characterized by the well-known complex periodic cross-striations. It has been suggested on the basis of this finding that histological differences between these fibre-types are conditioned, not by differences in their fibril-elements, but by the varying constitution of their cementing substances (Wassermann, 1956). For th [illegible] fibrils in connectior [illegible] regards the st [illegible] 1949, 1950). [illegible] *fibrils and a cementing substance typical for reticulin.*

Since we assume the formation of fibrils to be basically extracellular, it is hard to imagine how *identical* fibrils are formed in different tissues and organs out of different cementing substances. Here we have to ask whether the observation of identical cross-striations is a sufficient criterion of *identity of the fibrils.* One could, for instance, interpret the cross-striations merely as a structural characteristic common to all connective tissue fibrils. In that case, the cross-striations would not be the trade-mark for collagen

When one wants to
ron microscopy,
as in histology.
These can operate not only for the classification of individual con-

I should like to demonstrate the method of silvering collagenous

region of the dark band.

The contrasting strengthening of the dark bands by the silver
granules makes the transverse striation period of the fibrils appear

Katzenhofer (1953) and Pahlke (1954) for human tendon, and by
Linke (1955) for human skin. One could take the view that this
involves, strictly speaking, not an implantation of silver particles

If the silver were deposited on the surface of the fibrils, longitudinal
sections of collagenous fibrils would be free of silver unless a fibril

the fibrils to the polysaccharide in the coating of cementing substance. their
material ise a
tissue, th the
internal silv
Irving and
question w
fibril belongs to the cementing substance or whether it is to be re-

the second case, one would have to assume that the polysaccharide is part of the structural framework of the collagenous fibril, as the findings of Grassmann (1955) suggest.

The method devised by Irving and Tomlin (1954) for silvering the argyrophil fibrils of the spleen had already been described by von Herrath and Dettmer (1951). Silver particles were found to be

silver particles to accumulate on the surface of the dark band of the

On the other hand the collagenous fibrils,
ing, must be regarded as argyrophilic.

from the examples given that the varying methods for
en various types of
rther to apply the
various methods to a study of the development of connective tissue *fibres and their alterations throughout life* (Schwarz, 1953; Pahlke, 1954; Linke, 1955, Jahnke, 1956). The fibrils of a collagenous fibre change their mode of silvering during development, as I wish to show in the case of the sclera. In the early embryonic stages, during which the sclera is incidentally still transparent, one finds only fibrils with an irregular *external silvering* (*Fig. 3a*). This mode of

silvering is encountered in all embryonic connective tissue forn irrespective of the organ or tissue involved. In later embryon stages the sclera becomes opaque. Bound up with this change is a alteration in the mode of silvering of a proportion of the fibrils. Th silver particles arrange themselves on the fibril surface in group (Fig. 3*b*). The periodic external silvering corresponds to the mode of silvering of reticular fibrils. As development advances the external silvering of most fibrils changes to the periodic internal silvering that is typical for collagenous fibrils (Fig. 3*c*). In this preparation of *sclera* from a 5-year-old child, one also finds isolated fibrils with periodic or non-periodic external silvering. This varying result of the mode of silvering of the fibrils in separate stages of development permits the conclusion that the fibrils are subject to qualitative changes during their development. The final stage of this qualitative maturation is the internally silvered fibril in the tendinous organs. Unless the electron microscopic findings are to be in direct opposition to ordinary microscopic findings, one may regard only the internally silvered fibrils as collagenous in the strict sense. In many organs this final stage of maturation is not reached. One finds throughout the life-span, fibrils which may be regarded as an intermediate stage in this process of maturation. Thus one finds in the cornea fibrils whose mode of silvering corresponds throughout life to the early embryonic fibril. The fibrils of the cardiac valve (Jahnke, 1956) display the same behaviour. The fibrils from the spleen (von Herrath and Dettmer, 1951), from the arachnoid membrane, and from the lung interstitial tissue (Schwarz, 1955) have a regular external silvering, corresponding to the late embryonic stages described for sclera. This group could be classified as reticular fibrils; likewise, from the point of view of the maturation process described above, they could be called pre-collagenous fibrils. This qualitative process is to be interpreted as a method of differentiation of the inter-cellular substance. One could draw a parallel between this process and the change in solubility of the collagen with advancing age (Banfield, 1954), or between this process and certain chemical differences of collagens and procollagens (Grassmann and Kühn, 1955).

In every organ the intercellular substance reaches a definite differentiation level which, under normal conditions, is maintained throughout life. This status of differentiation once reached and then maintained would appear to be different for every organ and cor-



Fig 1

Plantar [illegible]

[illegible] irradiated by means of high-frequency resonance After the application of resonance, silvering according to Gomori s method

When one compares the development of distribution curves for a tendinous organ (Fig. 5) with the composite distribution curves of

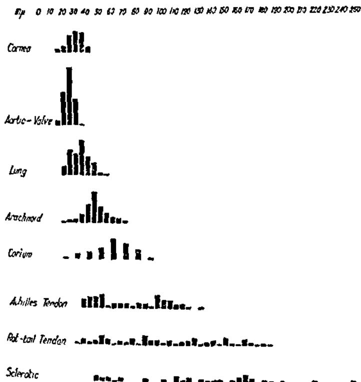
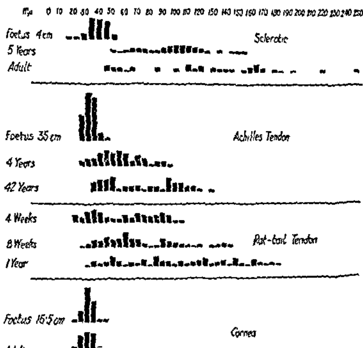


FIG. 4
Distribution-curves showing fibril-thickness of various intercellular substances, from connective tissue, from adult individuals. 200 fibrils were counted out in each case

various adult organs (Fig. 4), one finds the following results: the development of the Achilles tendon or of the sclera comprises a spectrum of


mμ 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250
Foetus 4cm
5 Years
Adult
Sclerotic
Foetus 35cm
Achilles Tendon
4 Years
42 Years
4 Weeks
8 Weeks
Rat-tail Tendon
1 Year
Foetus 16·5cm
Cornea

and differentiation are stopped at that point in time when the functional optimum of the respective intercellular substance is reached. In this way, for each organ a specific status of intercellular substance is reached which is appropriate to the function of the organ. A change in the state of the intercellular substance is possible in various ways In the case of intercellular substances whose fibrils do not reach, under normal conditions, the final stage of differentiation and growth, characterized by thick, internally silvered collagenous fibrils, further growth and differentiation in the direction of collagen are possible only under pathological conditions or in the event of senile alteration. An example of this is the corneal cicatrix (leu-:oma) whose distribution curve is presented in Fig. 5 Here one inds a further differentiation and also a growth of the fibrils (in -omparison with the arachnoid membrane of a 30-year-old) due to senile changes The possibility of further growth and differentiation of the fibrils is not present in those organs in which most of the fibrils have under normal conditions already arrived at the final stage. Here only regressive processes can lead to changes in the fibrils. As an example of this one might mention the skin whose fibrils grow thinner again in old age (Linke, 1955). But alongside this regression, there also occurs in the thick fibrils, in advanced age a certain degree of production of new intercellular substance and from this fresh formation of thin fibrils. Because of this, the range of fluctuation remains relatively great even in old age.

All of these senile or pathological changes, regardless of whether they consist of further differentiation producing increased thickness or regressive changes in those tissues incapable of further differentiation, impair the functioning of the tissue concerned.

Thus far I have kept the fibrils and their changes in the foreground of attention But the second component of a connective tissue fibre, the cementing substance, is also subject to qualitative and quantitative changes These changes are, however, difficult to understand with existing morphological methods, although a study of shadowed pictures of intercellular substance offers more hope of success. Here one can see, after appropriate preliminary treatment, a varying mass of fine coarsely granular material alongside the fibrils This substance appears to be partly dried up, and does not completely surround the fibrils. One finds a considerable mass of cementing substance in embryonic, arrested tissues or at embryonic differentiation levels of such tissues. As the fibrils grow and differentiate, the rela-

tive mass of cementing substance generally decreases. In the thick and differentiated fibrils of the tendon only traces of cementing substance are found between the fibrils. Only in the sclera is a

cementing substance for the fibrils. The affinity is reduced as differentiation progresses. In this connection I cite Fig. 1*a* and Fig. 2*a*. After identical preliminary treatment of both preparations, a difference can be seen, not only in the amount of cementing substance, but also in the way in which it adheres to the fibrils. The fibrils of plantar aponeurosis (Fig. 1*a*) lack a coating of cementing substance, most of this material lying free and dried up between the fibrils. The fibrils of the arachnoid membrane, on the other hand, show a distinct coating of cementing substance partly concealing their periodic cross-striations. Owing to this large amount of cementing substance, the fibre complex is much better preserved than in the plantar aponeurosis. Various connective tissue fibres can thus be distinguished by *the evaluation of shadowed fibril-preparations.*

... is available in that one can esti-
... on
... ds
(hyaluronidase) together with the periodic acid-silver reaction (Dettmer and Schwarz, 1954). Thus it can be shown that the polysaccharide content of the cementing substance is highest in embryonic tissue and that it falls as differentiation proceeds.

In a cross section of the connective tissue even when the cementing substance cannot be recognized, the fibres can be distinguished, and ... *relationships of fibrils to each*
... evaluation.
... each other,
whereas the fibrils of the ... b) appear to be loosely enclosed within the fibre. One must, however, imagine the spaces between the fibrils to be filled with cementing substance. Apparently the cementing substances are concealed by the impregnation material which remains in the section.

The identification of elastic fibres by means of the electron microscope is not difficult. They are easy to recognize in a section as

homogeneous, ramified or unramified bands which reveal no fibrillar structure under normal conditions. After extended 'fixation' in buffered osmic acid, the homogeneous appearance of the elastic fibre disappears, and then a net of filaments 70-100 Å thick appears. These filaments might be regarded as the proelastin which Hall and associates described (1952). The elastomucin appears to go into solution in buffered osmic acid (Dettmer, 1956). Further detailed observations are needed to determine whether the elastic fibres undergo a diminution of growth and of differentiation processes as do the other connective tissue fibres. Our experiments on the aorta are not yet finished.

I have tried to present the differences in connective tissue fibres which are observed with the electron microscope. The growth and differentiation of the intercellular components of connective tissue, on the evidence submitted would indicate the existence of a dynamic system, the conditioning factors for which are unknown to us. Research on these factors will only be possible when the cells are included in the treatment of the problem.

SUMMARY

Argyrophil and collagenous fibres consist of fibrils which are often invisible under the ordinary microscope, and of a cementing substance which occupies the spaces between the fibrils. The fibrils of both fibre-types display the well-known periodic cross-striation. Thus, they present the same appearance under the electron microscope. Most investigators regard this cross-striation as the 'fingerprint' for collagen; a precise distinction on this basis between the two fibre-types is not possible, however, with the electron microscope. Fibre thickness has, therefore, been adopted as the criterion for distinguishing reticular from collagenous fibrils, in order to establish a connection with histological observations. The increase in thickness signifies only a quantitative process and may be regarded as evidence of growth of the fibrils. The method of Gömori for silver-impregnation of the connective tissue, however, enables the electron microscopist to differentiate argyrophil from collagenous fibrils and fibres qualitatively. The collagenous fibrils, of the plantar aponeurosis for example, take up silver on the dark band of the cross-striation, thereby making the striations more prominent. This internal silvering is apparent not only on isolated fibrils but in

sectioned material as well. On a longitudinal section of a fibril 180 mµ. thick, one sees four to five granules lying in a row beside each other in the dark band of the fibril. On the other hand, in the case of argyrophil fibres the silver is found on the surface of the fibrils The silver granules are arranged in groups which correspond topographically to the dark bands of the fibrils. Thus the reticular fibril is characterized by a periodic surface silvering. The actual fibrils of the argyrophil fibres are argyrophobic and the fibrils of the collagenous fibres argyrophilic. A qualitative distinction between different fibrils and between the fibres is possible through the utilization of this characteristic affinity for silver. The internally silvered collagenous fibrils are first found in the connective tissue of the organs in question at a certain stage of development. Observations on the development of such connective tissue (sclera, Achilles tendon) reveal a differentiation which is visible in the electron microscope. The fibrils of the embryonic sclera show a completely random silvering on their surface. This manner of silvering is conditioned by the fibrils' coating of cementing substance. In the course of further development of the tissue, the silver granules arrange themselves on the surface of the fibrils in groups which correspond topographically to the reticulin of the histologist. As the differentiation proceeds the silver granules are increasingly impregnated in the dark band of the fibril. The silvering depends probably on carbohydrates Thus process of differentiation takes place ith all embryonic and/or newly formed fibrils, but all connective ssues do not reach the final stage (internal silvering). On the conary, many tissues remain at an intermediate stage of the differentiaon (reticular connective tissue, interstitial tissue of the lung). The reticular stage is in fact never reached by bradytrophic tissue of he cornea during normal development. A further differentiation, beyond that which is typical for each connective tissue, signifies an impairment of function. For example, the cornea displays a furthe differentiation of the intercellular substance which is bound up wit loss of transparency as in leucoma. In the parenchymatous organs, further differentiation of the interstitial tissue implies a change whic we know as sclerosis The cementing substance, which is foun between the fibrils, participates in the differentiation process of t fibre. Its relative amount decreases during the process of different tion. Its quality changes also, even though this is difficult to int pret morphologically. One must, for genetic reasons, regard

fibre forms of the reticulin and collagen as an extended spectrum, which the reticular and collagenous fibre signifies only a roughl schematic classification of fibre forms. Morphologically the elasti fibre assumes an exceptional position. It consists of filaments and cementing substance. The elastic fibres appear homogeneous in section inasmuch as the filaments are entirely masked by the cementing substance. These filaments may, however, be demonstrated by using special methods, but more exhaustive observations are needed to determine whether the elastic fibres go through a process of differentiation similar to that of the other connective tissues.

GROUP DISCUSSION

Dr. Banga asked whether silver binding depends on the histidine content of collagen as described by Dr Grassmann. Dr Orekhovitch had stated that procollagen contained three times more histidine than native collagen. She had also found that the silver binding of procollagen was three times more than that found in native collagen fibrils. After extraction of mucopolysaccharide from the native collagen she found that the silver-binding capacity of the fibres dropped also. Potassium iodide relaxed fibrils (metacollagen) have a much smaller silver-binding capacity than the native fibre. Does silver binding depend on the mucopolysaccharide or the histidine content of the fibril?

Dr Grassmann replied that the silver binding he described in 1951 and the silver staining described here today were two quite different things. One is a complex binding of the silver ions and the other is a deposition of silver metal particles. They had found a stoichiometric correlation between the histidine content of collagen and the quantity of silver ions bound by collagen. They had found exactly the same histidine content in procollagen as in native collagen. In two samples of collagen (purified by salt extraction and followed by treatment with calcium hydroxide and trypsin) the percentage of histidine-N of total nitrogen obtained was 1.52 per cent and 1.48 per cent. In two samples of procollagen the percentages of nitrogen obtained were 1.35 and 1.41. The use of stereoscopic electron microscopic pictures can distinguish whether particles of silver lie inside or outside small collagen or reticulin fibrils. Work had already been started on Achilles tendon and long-spacing fibrils and they had demonstrated quite definitely that some silver particles lie within the fibril.

Dr Schwarz felt that most histological stains were non-specific and that of all the various silver impregnations, the Gömöri method proved

[illegible]

fibrils and a lot of cementing substance which masked the fibrils, thus [illegible]

up silver in an irregular manner like an embryonic tissue (bradytrophic tissue).

Dr. Reed pointed out that the wide differences in fibril width and silver-staining properties that Dr. Schwarz had demonstrated seemed to be accompanied also by a wide difference in their physical and chemical properties in this reticulin-collagen system. For instance, they all shrank and responded to different enzymes at different rates and he thought it might be possible that, when the biochemist was extracting material from this system, he was dealing with fibres that differed both in age and [illegible] composition.

[illegible] ...hwarz as to [illegible] ...idered that [illegible] not necessarily the best but that the best method [illegible]

grains in silver-stained material was similar to the photographic process which is catalysed by sulphur compounds and reducing substances of low molecular weight. If that were so, it was possible that the differences of various fibres from different tissues might be due to the presence or absence of these reducing agents near the fibres. It would then be unnecessary to assume that the collagen varied in itself from place to place.

Dr Schwarz replied that the size of the silver granules depends on the site of deposition. As to relation to photographic process, there are certain parallels, though the processes are not exactly identical. In reply to Dr. Gross's objection, Dr Schwarz answered that he had taken precautions mainly consisting in keeping the beam intensity low.

ELECTRON MICROSCOPE AND CHEMICAL STUDIES OF THE CARBOHYDRATE GROUPS OF COLLAGEN

W. GRASSMANN, U. HOFMANN, K KUHN, H HORMANN, H. ENDRES AND K. WOLF

The opinion that carbohydrate components, which are known to be elements of the ground substance of connective tissue, play an essential role in the formation and in the structure of collagenous fibrils, is based on the following findings:

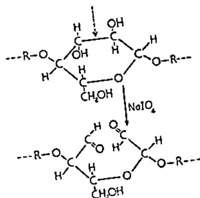
(1) The customary standard methods for the analysis of glycoproteins such as, for example, the orcinol reaction (Sørensen and Haugaard, 1933), the anthrone reaction (Dreywood, 1946), the Elson-Morgan reaction (Elson and Morgan, 1933; Blix, 1948, Boas, 1953), etc., have shown that all collagenous substances contain small amounts – not more than 1 per cent – of carbohydrates (Grassmann and Schleich, 1935; Beck, 1941; Bangle and Alford, 1954, Moss, 1955). Such carbohydrates were isolated and identified by means of chromatographic methods, and the following were described as components of collagen: glucose (Grassmann and Schleich, [illegible] 5), mannose (Gross, [illegible] legg, Eidinger and [illegible]

(2) It has further been shown that the formation of some distinct types of fibrils in collagen solutions is caused and controlled by small amounts of accompanying substances containing acidic carbohydrates. The presence of hyaluronic acid, α_1-glycoprotein or heparin results in the formation of 'long-spacing collagen' (Highberger, Gross and Schmitt, 1951), whereas nucleic acid or ATP form 'long-spacing segmented collagen' (Schmitt, Gross and Highberger, 1953). The presence or absence of such carbohydrate [illegible] factor for the solubility of [illegible]

[illegible] histology, the PAS reaction (McManus, 1940), and the periodate

silver reaction (Dettmer and Schwarz, 1952/53) are apparently carbohydrate reactions. The two latter ones are certainly due to aldehyde groups formed by the action of sodium periodate.

FORMULA I



These results were further pursued in two directions: (*a*) Schwa and Dettmer (Schwarz, 1955; Dettmer, 1955) have shown that t silver staining of collagen, in particular the method employi periodate-silver urotropin (Dettmer and Schwarz, 1952/53), c be used to trace carbohydrates in the electron microscope or – to p it more cautiously – to trace substances that react to sodium per date by forming aldehydes. Pahlke (1954), by using this meth was able to show that the deposits of silver grains in embryo sinews occur mainly in the amorphous ground substance. V increasing age these silver deposits in the interstitial subst gradually decrease and the silver grains are found in fine dispe on the surface of the fibrils, and in adults such deposits occur regular order in the dark bands of the cross striation. Accordi Schwarz and Pahlke this periodic 'intrafibrillic silver staining' cates the maturity of a fibril. (*b*) In our laboratory, on the hand, we were able to show (Grassmann and Kühn, 1955; mann, Hörmann and Klenk, not published) that the various k collagens are dissolved and disintegrated if treated with s periodate or similar reagents for some length of time. In this p α-amino groups of glycine, alanine, valine and no others are Their quantity is equivalent to an average chain length of a

amino acids in the case of the disintegration product obtained from collagen and of 18 amino acids in the case of procollagen.

We have studied the dissolution of collagen fibrils under the action of periodate through the electron microscope by employing the silver-staining method (Kühn, Hofmann and Grassmann, 1956). Figs. 1, 2, 3, 4 show the action of a 4 per cent sodium periodate solution on Achilles tendon fibril in four subsequent stages, namely after a quarter of an hour, after one hour and a half, after 18 hours, and after 43 hours, whereupon, in each case, the silver-staining method was employed in the usual manner. Fig. 1 shows a normal order of silver deposits in a collagenous fibril after 15 minutes' treatment with periodate.

Even after only one hour and a half (Fig. 2) a splitting off of fibril parts can be observed. The silver deposits still give the impression of being fairly periodic. The longer the oxidation process went on, the darker the silver solution became, which again indicates that part of the aldehyde-bearing material dissolves and forms silver grains in the solution. After 18 hours (Fig 3) the silver grains are found to have become progressively smaller in size but are still in periodic order. It is remarkable that now, under the relatively thinner silver deposits, the normal cross striation of the fibril begins to show, but because of the lack of any phosphotungstic staining such cross striation is not sufficiently contrasty in these experiments. After 43 hours (Fig. 4) the major parts of fibrillic substance are dissolved The few remaining parts, however, still reveal periodic silver deposits.

Procollagen, as defined by Orekhovitch, is dissolved much more rapidly. The results obtained after silver staining are essentially the ... Achilles tendon Sodium periodate also ... of time.

ve

is

al

(1) Are the parts in which silver ... is deposited, i.e. the classi- with those in which phosphotungstic acid is deposited, i.e. the classical D-bands?

(2) Is periodic silver staining a general and reliable characteristic of mature fibrils, and irregular staining one of immature fibrils? ... actually take place within ... periodic silver staining ... cross section?

2) it was necessary

1955) we have shown examples of highly subdivided fibrils taken from cats' tails and having 10 and more cross striations and of collagenous fibrils of cervical ligament in man. A highly subdivided fibril from a man's Achilles tendon is shown in Fig. 5. It was stained with phosphotungstic acid and reveals 13 cross striations. Fig. 1 *shows the same substance after* action of hyaluronidase and subsequently of periodate and silver. Periodic deposits with up to three bands per period can be observed. It is remarkable that the periodic silver deposits neither disappear nor diminish after treatment with hyaluronidase, on the contrary, we often find clearer *pictures, since any deposits of carbohydrate-containing ground* substance on the surface are removed.

The Achilles tendon of a human embryo (total length 14 inches) shows 10 intra-periods after having been treated with phosphotungs- [illegible] here is too [illegible] *Weak, but* [illegible] aining was [illegible]

It is not an established rule that the periodic silver deposits always occur in the dark bands of fibrils. Fig. *6a* is a photograph stained *with a combination of silver and phosphotungstic acid* showing with sufficient clarity both the periodic silver deposits and the normal cross striation. Surprisingly enough we find an illustrative example of both possibilities. In one fibril the silver deposits can be clearly seen in the dark bands, in the other in the light bands.

Reticular fibrils from spleen are thin, show poor contrast and usually contain considerable amounts of interstitial substance. We have not yet heard of any photographs of highly subdivided cross striation. Fig. 7 shows a fibril with 10 *intra-periods after treatment* with phosphotungstic acid, whose intensity is slightly different from the normal. In the case of silver staining, the presence of considerable amounts of amorphous interstitial substance often proves disturbing, but such disturbances can largely be eliminated by previous *treatment with trypsin or hyaluronidase*. After such treatment we were able in a large number of cases to obtain periodic silver deposits in these materials also (Fig. 8).

In procollagen, highly subdivided cross striation has not been observed until recently (Kühn, Hofmann and Grassmann, 1956). The cross striation of procollagen is in part entirely identical with

procollagen fibrils without any exception showed regular and very distinct intrafibrillar silver staining (Fig. 10). Three intra-periods

(Schwarz

centre, into the neighbouring area. Could this nucleus inducing the formation of a fibril be an acid glycoprotein?

The silver staining of long-spacing fibrils, which has not been described so far, shows marked periodic deposits of silver grains. Up to 6 bands per period are discernible. Fig. 11 shows photographs taken of the same product, in one case not treated, in the other treated with periodate silver, and subsequently brought into register with each other. Such register can be clearly established because there are preparations in which the normal cross striation is evident through the silver deposits. A comparison reveals that the middle band, which is most distinct, both with and without phosphotungstic acid staining, by no means shows any outstanding

amorphous carbohydrate-containing ground substance *in vivo*, or

the formation of specific collagen structures in the presence of certain polysaccharide fractions *in vitro*, or anyone studying in the electron microscope the dissolution of collagen under the action of periodate or the deposition of silver, will necessarily come to the conclusion that carbohydrate groups (or, to put it in a more cautious way, groups that react with periodate, thereby forming aldehydes) play an important part in the structure of collagenous fibrils.

But I should also like to draw attention to the somewhat difficult nature of this problem if we approach it from another angle, for example, by applying the classical methods for the determination of carbohydrates in proteins, such as the orcinol reaction, or the anthrone reaction which once helped us to establish the existence of carbohydrates in collagenous fibrils (Grassmann and Schleich, 1935).

TABLE I

CHANGE IN CARBOHYDRATE CONTENT OF CALF PROCOLLAGEN DURING RECRYSTALLIZATION

Sample	*Per cent Hexose*	*Per cent Hexosamine*
Procollagen impure	0 91	0 130
once recrystallized	0 84	0 085
twice recrystallized	0 73	0 011

When using these methods we found slightly less than 1 per cent hexoses and small varying amounts of glucosamine in collagen preparations. In previous experiments (Schneider, 1949) we tried to concentrate these carbohydrates in order to gain an insight into their composition and chemical linkage. At that time, this was done in accordance with the methods worked out by Rimington *et al.* (Rimington, 1931) for the examination of glycoproteins. partial hydrolysis with barium hydroxide, and subsequent precipitation with lead salts and mercuric salts We were able to obtain products with a carbohydrate content of about 50 per cent, the yield being

these products are not of a homogeneous nature and, even worse, that their composition differs according to the nature of the starting material employed. Electrophoresis established that these products can be divided into two fractions, one of which contains glucose, galactose, mannose and glucosamine in a molecular ratio of roughly 1:1·1:1, whereas the oth
1:1, but is free of manno
then was cattle corium
calcium hydroxide had not been specially purified, therefore it cannot be said with certainty whether the small amounts of carbohydrates that were isolated in that way came from the collagen itself or from impurities in the starting substance.

The purification of the native collagen is a rather problematic affair, even though the material may, for example, be successively treated with salt solutions, calcium hydroxide and trypsin (Grassmann, Janicki and Schneider, 1937).

TABLE II

HEXOSE CONTENT OF SOME SAMPLES OF COLLAGEN AND PROCOLLAGEN
(Anthrone method)

Material	Origin	*Per cent Hexose*
Procollagen I rough	calf	0.91
Procollagen I once recrystallized	calf	0.84
Procollagen I twice recrystallized	calf	0.73
Procollagen II once recrystallized	cow	2.71
Procollagen III once recrystallized	cow	1.10
Procollagen III twice recrystallized	cow	0.98
Collagen pure*	cow	0.42

* Purification by extraction with NaCl, $Ca(OH)_2$ and treatment with trypsin (Grassmann, Janicki and Schneider, 1937)

Procolla
ko
pu
shows that glucosamine is not an essential constituent of collagen fibrils *repeated reconstitution eliminates it almost entirely. The* hexoses, however, unlike glucosamine, decrease only slightly in



Fig 7
Reticular fibril from cat's spleen treated with hyaluronidase and subsequently with phosphotungstic acid *Electron microscope magnification* × 51,600 10 cross striations

Fig 8
Reticular fibril from cat's spleen treated with trypsin Periodate-silver-urotropin method (15 minutes' treatment with $NaIO_4$ and *8 hours' silver staining) Electron microscope* magnification × 12,900

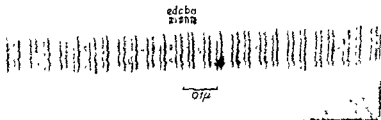


Fig. 9
Fibril of procollagen, treated with phosphotungstic acid. Electron microscope magnification × 51,600. 13 cross striations.

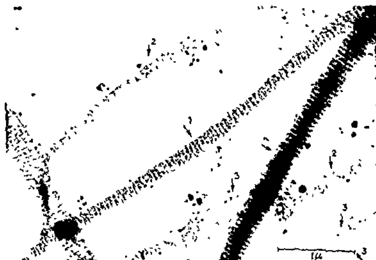


Fig. 10
Fibrils of procollagen, treated with trypsin, periodate-silver-urotropin method (15 minutes treatment with [illegible] Up to three silver [illegible] silver [illegible]

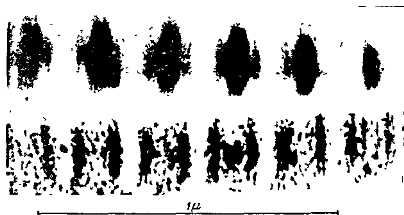


FIG 11

Long-spacing fibrils prepared in an acidic solution of procollagen with α_1-glyco-protein treated with hyaluronidase Electron microscope magnification × 12,900

Top without staining
Bottom after treatment with periodate-silver
Subsequently brought into register with each other
Periods approx 2000 Å

treated collagen is treated with trypsin, a small amount (0.8 per cent) of a residue is left that is resistant to tryptic digestion. This residue contains more than 20 per cent carbohydrates (Grassmann, Hannig and Wolf, unpublished).

Considering such hexoses as may be determined by means of the customary standard methods it is obvious that hardly more than 0.5 per cent can ever have any true chemical linkage to protein. Such linkage could be possible in the following ways:

(1) N-glycosidic linkages between sugar and the amino groups of protein.

(2) O-glycosidic linkages to the hydroxy groups of hydroxy-amino acids.

(3) Ester linkages between the alcoholic hydroxy groups of sugars and the carboxyl groups of protein.

N-glycosidic linkages between sugars and amino acids are extremely unstable towards acids and bases (Micheel and Klemer, 1951, 1956), and N-glycosides form osazones with phenylhydrazine without any acid hydrolysis being necessary (Grassmann, Hörmann and Hafter, in press). The carbohydrates of collagen, however, form osazone only after having been split off by means of acid under such conditions as are required for the splitting of normal O-glycosidic linkages. A number of other findings, in particular also methylation experiments, indicate that part of the carbohydrate that is determinable by means of anthrone must be polysaccharides.

The existence of ester linkages in collagen and procollagen, on the other hand, must be considered highly probable because of the latter's behaviour towards lithium boronhydride (Grassmann, Endres and Steber, 1954, Grassmann and Endres, unpublished). According to all the previous findings, lithium boronhydride is not able to reduce free carboxyl groups in peptides and proteins whereas after esterification it reduces them easily to amino alcohols. It also does not split off peptide linkages (Hormann, Grossmann, Wünsch and Preller, 1956). This makes it possible to determine the quantity of C-terminal amino acids in peptides and proteins. In the case of collagen and procollagen substantial amounts of amino alcohols of certain amino acids are obtained without previous esterificatio-



FIG 11

Long-spacing fibrils prepared in an acidic solution of procollagen with α_1-glyco-protein treated with hyaluronidase Electron microscope magnification × 12 900

treated collagen is treated with trypsin, a small amount (0.8 per cent) of a residue is left that is resistant to tryptic digestion. This residue contains more than 20 per cent carbohydrates (Grassmann, Hannig and Wolf, unpublished).

Considering such hexoses as may be determined by means of the customary standard methods it is obvious that hardly more than 0.5 per cent can ever have any true chemical linkage to protein. Such linkage could be possible in the following ways:

(1) N-glycosidic linkages between sugar and the amino groups of protein.

(2) O-glycosidic linkages to the hydroxy groups of hydroxy-amino acids.

(3) Ester linkages between the alcoholic hydroxy groups of sugars and the carboxyl groups of protein.

N-glycosidic linkages between sugars and amino acids are extremely unstable towards acids and bases (Micheel and Klemer, 1951, 1956), and N-glycosides form osazones with phenylhydrazine without any acid hydrolysis being necessary (Grassmann, Hormann and Hafter, in press). The carbohydrates of collagen, however, form osazone only after having been split off by means of acid under such conditions as are required for the splitting of normal O-glycosidic linkages. A number of other findings, in particular also methylation experiments, indicate that part of the carbohydrate that is determinable by means of anthrone must be polysaccharides.

The existence of ester linkages in collagen and procollagen, on the other hand, must be considered highly probable because of the latter's behaviour towards lithium boronhydride (Grassmann, Endres and Steber, 1954, Grassmann and Endres, unpublished). According to all the previous findings, lithium boronhydride is not able to reduce free carboxyl groups in peptides and proteins whereas after esterification it reduces them easily to amino alcohols. It also loes not split off peptide linkages (Hormann, Grossmann, Wünsch nd Preller, 1956). This makes it possible to determine the quantity f C-terminal amino acids in peptides and proteins. In the case of ollagen and procollagen substantial amounts of amino alcohols of rtain amino acids are obtained without previous esterification.

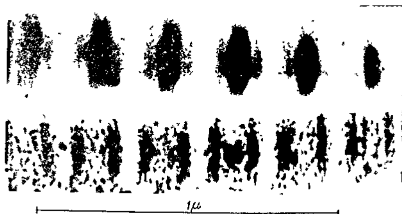


Fig 11

Long-spacing fibrils prepared in an acidic solution of procollagen with α_1-glyco-protein treated with hyaluronidase Electron microscope magnification $\times$ 12,900

Top without staining
Bottom after treatment with periodate-silver
Subsequently brought into register with each other
Periods approx 2000 Å

treated collagen is treated with trypsin, a small amount (0.8 per cent) of a residue is left that is resistant to tryptic digestion. This residue contains more than 20 per cent carbohydrates (Grassmann, Hannig and Wolf, unpublished).

Considering such hexoses as may be determined by means of the customary standard methods it is obvious that hardly more than 0.5 per cent can ever have any true chemical linkage to protein. Such linkage could be possible in the following ways:

(1) N-glycosidic linkages between sugar and the amino groups of protein.

(2) O-glycosidic linkages to the hydroxy groups of hydroxy-amino acids.

(3) Ester linkages between the alcoholic hydroxy groups of sugars and the carboxyl groups of protein

N-glycosidic linkages between sugars and amino acids are extremely unstable towards acids and bases (Micheel and Klemer, 1951, 1956), and N-glycosides form osazones with phenylhydrazine without any acid hydrolysis being necessary (Grassmann, Hormann and Hafter, in press). The carbohydrates of collagen, however, form osazone only after having been split off by means of acid under such conditions as are required for the splitting of normal O-glycosidic linkages. A number of other findings, in particular also methylation experiments, indicate that part of the carbohydrate that is determinable by means of anthrone must be polysaccharides.

The existence of ester linkages in collagen and procollagen, on the other hand, must be considered highly probable because of the latter's behaviour towards lithium boronhydride (Grassmann, Endres and Steber, 1954; Grassmann and Endres, unpublished). According to all the previous findings, lithium boronhydride is not able to reduce free carboxyl groups in peptides and proteins whereas after esterification it reduces them easily to amino alcohols. It also does not split off peptide linkages (Hörmann, Grossmann, Wunsch nd Preller, 1956). This makes it possible to determine the quantity f C-terminal amino acids in peptides and proteins. In the case of ollagen and procollagen substantial amounts of amino alcohols of rtain amino acids are obtained without previous esterification.

м



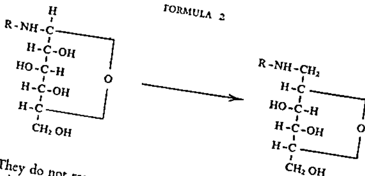
Fig 11

Long-spacing fibrils prepared in an acidic solution of procollagen with α_1-glyco-protein treated with hyaluronidase Electron microscope magnification × 12,900

Top without staining
Bottom after treatment with periodate-silver
Subsequently brought into register with each other
Periods approx 2000 Å

and Kuhn, 1955; Grassmann, Hormann and Klenk, unpublished).
My colleague, Professor Zahn (Zahn, 1956), suggested that hydroxylysine, present in collagen in a quantity of 0.7 per cent, also in peptide linkage, might fulfil all the requirements for an action of sodium periodate, it must split off formaldehyde if disintegrated. It is true that formaldehyde is formed if sodium periodate acts on procollagen, and its amount is in conformity with the amount of hydroxylysine present (Grassmann, Hormann and Fries, unpublished) But the periodate consumption is far higher than would be justified by the sum of hydroxylysine and such carbohydrate as can be determined by anthrone, quite apart from the fact that an oxidation of the side chains of hydroxylysine would hardly account for a disintegration of collagen and procollagen by sodium periodate, and would certainly not explain the formation of α-amino groups.
Compounds of such kind as are formed from N-glycosides by means of the Amadori rearrangement (Amadori, 1925) have recently been isolated from biological sources

FORMULA 2



They do not react with anthrone (Borsook, Abrams and Lowy, 955) and we also found that they produce osazone (Grassmann, Hormann and Hafter, in press) under the action of phenyl hydrazine ithout any previous acid hydrolysis being required. It is therefore ghly improbable that such anthrone-negative carbohydrate groups e the cause of the reaction with periodate.
have considered it my task to show you how many questions still not be answered in respect of the groupings of a carbohydrate are in collagen. Formerly, the main difficulty and the crux of the

[illegible] carbohydrates that can be traced [illegible] by means of periodate that were observed in our laboratory? I do not think so.

It has already been mentioned that, when splitting collagen or procollagen with periodate or phenyl iodosoacetate α-amino groups are set free whose quantity is equivalent to a medium chain length of 24 amino acids in the case of collagen and to 18 amino acids in the case of procollagen. The same chain lengths (Grassmann, Endres and Steber, 1954; Grassmann and Endres, unpublished), however, are found in the reductive disintegration of the presumed ester linkages by means of lithium boronhydride. But if there are really any 'weak joints' in the peptide chains of collagen that can be attacked by sodium periodate by oxidation and by lithium boronhydride by reduction, and if a disintegration of these 'weak joints' results in chain lengths of 24 or 18 amino acids respectively, it is obvious that, on the average, every 18 or 24 amino acids there must be such a 'weak joint'; and if these 'weak joints' were actually hexoses, at least 4-5 hexoses must exist per 100 mol of amino acids, which is equivalent to roughly 7 per cent by weight of [illegible] by means of the [illegible] uld contain a certain percentage of a carbohydrate-like substance of unknown quality. The amino acid analysis of collagen covers about only 94 per cent in weight, but nearly 100 per cent of the total N, as was found by Bowes, Elliott and Moss (1955) and can be confirmed by us (Grassmann, Hanning and Plockl, 1955). The periodate con- [illegible] defined [illegible] te content were about 0·7 per cent (Grassmann, [illegible] s, unpublished).

Are there any other known groups in collagen that may be responsible for such periodate consumption and for the dissolution of collagen and procollagen in sodium periodate?

There is no reason why the two hydroxy-amino acids, serine and threonine, should be affected by periodate, if they are part of a peptide chain. And, indeed, the quantity of these amino acids is hardly ever smaller after disintegration with periodate (Grassmann

and Kühn, 1955, Grassmann, Hormann and Klenk, unpublished).

My colleague, Professor Zahn (Zahn, 1956), suggested that hy-

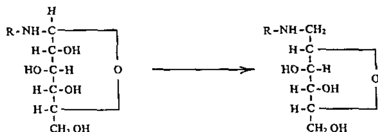
procollagen, and its amount is in conformity with the amount of hydroxylysine present (Grassmann, Hormann and Fries, unpublished). But the periodate consumption is far higher than would be justified by the sum of hydroxylysine and such carbohydrate as can be determined by anthrone, quite apart from the fact that an oxidation of the side chains of hydroxylysine would hardly account for a disintegration of collagen and procollagen by sodium periodate, and would certa

Compounds

means of the A

cently been isolated from biological sources

FORMULA 2



They do not react with anthrone (Borsook, Abrams and Lowy, 1955) and we also found that they produce osazone (Grassmann, Hormann and Hafter, in press) under the action of phenyl hydrazine without any previous acid hydrolysis being required. It is therefore highly improbable that such anthrone-negative carbohydrate groups are the cause of the reaction with periodate.

I have considered it my task to show you how many questions still cannot be answered in respect of the groupings of a carbohydrate nature in collagen Formerly, the main difficulty and the crux of the

But are those small quantities of carbohydrates that can be traced by the anthrone and similar reactions really essential for collagen? Are they responsible for the dissolution and disintegration of collagen by means of periodate that were observed in our laboratory? I do not think so.

It has already been mentioned that, when splitting collagen or procollagen with periodate or phenyl iodosoacetate α-amino groups are set free whose quantity is equivalent to a medium chain length of 24 amino acids in the case of collagen and to 18 amino acids in the case of procollagen. The same chain lengths (Grassmann, Endres and Steber, 1954; Grassmann and Endres, unpublished), however, are found in the reductive disintegration of the presumed ester linkages by means of lithium boronhydride. But if there are really any 'weak joints' in the peptide chains of collagen that can be attacked by sodium periodate by oxidation and by lithium boronhydride by reduction, and if a disintegration of these 'weak joints' results in chain lengths of 24 or 18 amino acids respectively, it is obvious that, on the average, every 18 or 24 amino acids there must be such a 'weak joint'; and if these 'weak joints' were actually hexoses, at least 4-5 hexoses must exist per 100 mol of amino acids, which is equivalent to roughly 7 per cent by weight of carbohydrate. The hexoses that can be ascertained by means of the anthrone reaction are one-tenth of this amount.

This indicates that collagen and procollagen should contain a certain percentage of a carbohydrate-like substance of unknown quality. The amino acid analysis of collagen covers about only 94 per cent in weight, but nearly 100 per cent of the total N, as was found by Bowes, Elliott and Moss (1955) and can be confirmed by us (Grassmann, Hanning and Plöckl, 1955). The periodate consumption of procollagen which does not lead to any sharply defined endpoint, is substantially higher than it should be if the carbohydrate content were about 0.7 per cent (Grassmann, Hörmann and Fries, unpublished).

Are there any other known groups in collagen that may be responsible for such periodate consumption and for the dissolution of collagen and procollagen in sodium periodate?

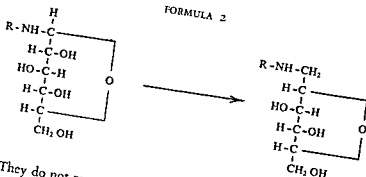
There is no reason why the two hydroxy-amino acids, serine and threonine, should be affected by periodate, if they are part of a peptide chain. And, indeed, the quantity of these amino acids is hardly ever smaller after disintegration with periodate (Grassmann

and Kuhn, 1955, Grassmann, Hormann and Klenk, unpublished).

My colleague, Professor Zahn (Zahn, 1956), suggested that hydroxylysine, present in collagen in a quantity of 0.7 per cent, also in peptide linkage, might fulfil all the requirements for an action of sodium periodate; it must split off formaldehyde if disintegrated. It is true that formaldehyde is formed if sodium periodate acts on procollagen, and its amount is in conformity with the amount of hydroxylysine present (Grassmann, Hormann and Fries, unpublished). But the periodate consumption is far higher than would be justified by the sum of hydroxylysine and such carbohydrate as can be determined by anthrone, quite apart from the fact that an oxidation of the side chains of hydroxylysine would hardly account for a disintegration of collagen and procollagen by sodium periodate, and would certainly not explain the formation of α-amino groups.

Compounds of such kind as are formed from N-glycosides by means of the Amadori rearrangement (Amadori, 1925) have recently been isolated from biological sources.

FORMULA 2



They do not react with anthrone (Borsook, Abrams and Lowy, 1955) and we also found that they produce osazone (Grassmann, Hormann and Hafter, in press) under the action of phenyl hydrazine without any previous acid hydrolysis being required. It is therefore highly improbable that such anthrone-negative carbohydrate groups are the cause of the reaction with periodate.

I have considered it my task to show you how many questions still cannot be answered in respect of the groupings of a carbohydrate nature in collagen. Formerly, the main difficulty and the crux of the

But are those small quantities of carbohydrates that can be traced by the anthrone and similar reactions really essential for collagen? Are they responsible for the dissolution and disintegration of collagen by means of periodate that were observed in our laboratory? I do not think so.

It has already been mentioned that, when splitting collagen or procollagen with periodate or phenyl iodosoacetate α-amino groups are set free whose quantity is equivalent to a medium chain length of 24 amino acids in the case of collagen and to 18 amino acids in the case of procollagen. The same chain lengths (Grassmann, Endres and Steber, 1954; Grassmann and Endres, unpublished), however, are found in the reductive disintegration of the presumed ester linkages by means of lithium boronhydride. But if there are really any 'weak joints' in the peptide chains of collagen that can be attacked by sodium periodate by oxidation and by lithium boronhydride by reduction, and if a disintegration of these 'weak joints' results in chain lengths of 24 or 18 amino acids respectively, it is obvious that, on the average, every 18 or 24 amino acids there must be such a 'weak joint'; and if these 'weak joints' were actually hexoses, at least 4-5 hexoses must exist per 100 mol of amino acids, which is equivalent to roughly 7 per cent by weight of carbohydrate. The hexoses that can be ascertained by means of the anthrone reaction are one-tenth of this amount.

This indicates that collagen and procollagen should contain a certain percentage of a carbohydrate-like substance of unknown quality. The amino acid analysis of collagen covers about only 94 per cent in weight, but nearly 100 per cent of the total N, as was found by Bowes, Elliott and Moss (1955) and can be confirmed by us (Grassmann, Hanning and Plockl, 1955). The periodate consumption of procollagen which does not lead to any sharply defined endpoint, is substantially higher than it should be if the carbohydrate content were about 0.7 per cent (Grassmann, Hörmann and Fries, unpublished).

Are there any other known groups in collagen that may be responsible for such periodate consumption and for the dissolution of collagen and procollagen in sodium periodate?

There is no reason why the two hydroxy-amino acids, serine and threonine, should be affected by periodate, if they are part of a peptide chain. And, indeed, the quantity of these amino acids is hardly ever smaller after disintegration with periodate (Grassmann

GROUP DISCUSSION

DR REED inquired about the exact conditions of Dr. Grassmann's experiments with periodate as regards pH, temperature, etc DR. GRASSMANN replied that 4 per cent periodate was used at pH values of 7 to 8 and phenyl-iodoso-acetate at pH 3 4 and the temperature was 40° C. Controls were done without periodate.

DR. D. S JACKSON stated that in his experiments on the effects of periodate on tendon, solution of the collagen depended entirely on the presence of swelling. If approximately 2M sodium chloride were included in the reaction mixture, then the fibres could be left almost indefinitely without any dissolution taking place at all. This suggested that whatever the periodate was reacting with, it might be a cross-linkage [illegible]

then form a type of gelatin

DR. GRASSMANN recalled that in his electron micrographs collagen [illegible]

was obtained

DR. GRASSMANN replied that for procollagen 60 per cent was dissolved within half a day, but collagen took longer.

DR. MEYER recalled that periodate was originally introduced into carbohydrate chemistry splitting glycol groups with the production of aldehyde, but this was done under certain defined conditions. In an unknown excess of periodic acid, such as used in the experiments described by Dr Grassmann, there was an indiscriminate splitting of C-C bonds which had no relation to this glycol structure. He wondered whether one could draw any conclusions as to the action of the periodate under the experimental conditions described Could Dr. Grassmann [illegible]

proline. There was no disappearance of amino acids under the action of periodate.

DR NEUBERGER felt very doubtful about the application of periodic acid in the way Dr Grassmann had suggested and he wished to support Dr. Meyer. It was quite clear that a cis-glycol or an alpha-amino beta-hydroxy-amino acid is split very quickly by periodic acid, but he felt that peptide linkages might be broken after long exposure to a large excess of

problem seemed to be that the carbohydrate content of the collagen fibril was small, and that it was difficult to decide whether such carbohydrate content was part of collagen itself or due to any impurities of the amorphous ground substances. At present I am inclined to see the crucial point of the problem in the fact that the groups responsible for the histological and electron microscope silver staining and the disintegration by periodate are not identical with the ones that can be traced by means of the hitherto customary carbohydrate reactions. It will be necessary, and we are at present trying, to explore these still unknown groupings in the disintegration products of collagen.

the binding capacity of collagen for co-ordination compounds, reacting by hydrogen bonding on the keto-imide group of collagen (mimosa tannins), is more than doubled. Also increased reactivity of the treated collagen by means of its anionic and cationic groups is observed. The periodate appears to rupture the stabilizing cross-links of the protein, making new hydrogen bonding loci available for reaction (on the -CO. NH- linkages mainly) However, the cleavage of some keto-imide bonds of the backbone is indicated to occur. According to Grassmann and Kuhn the amino acid composition of collagen is practically unaffected by the periodate treatment Sodium perchlorate shows exceedingly strong lyotropic effects on collagen also, as evident by the destruction of collagen, impaired thermal stability, and drastically increased co-ordinate reactivity. These effects occur very suddenly on raising the concentration of the perchlorate solution above one molar It looks like the periodate-collagen reaction is very complicated, involving far-going alterations of the protein units, apart from the effect on the carbohydrate component which reaction probably is of minor importance.

periodate. Could there not be a very small amount of destruction? Another point was the presence of small amounts of tyrosine (no one had ever obtained a collagen free from tyrosine), the molecules of which should be attacked by periodate.

[illegible]

oxidized as readily as the sugar to yield aldehyde groups to react with silver salts

DR GRASSMANN said it is indeed so that hydroxylysine is the only amino acid which fits the conditions for attack by periodate. When bound in the peptide chain serine and threonine *should not be attacked within the limitations* stated by Dr Meyer. In his own experiments, formaldehyde was set free in a quantity exactly equivalent to the hydroxylysine content. Thus, hydroxylysine is certainly attacked. But why should the peptide *link break and an alpha amino group result if anything happens to the* side chain of hydroxylysine?

[illegible] had considered the possibility

[illegible]

situated sulphur containing compounds (which can catalyse photographic processes at least) and the small number of such residues could explain the cross bands described by Dr Grassmann

Dr Gustavson mentioned some work he had done on the effect of

[illegible]

5.0, lasted for 5-7 days, solutions of various concentration of periodate being employed NaIO₄-solutions less than 0 25 molar had no appreciable solubilizing effect, neither did they lower the shrinkage temperature of collagen and its co-ordination capacity (hydrogen bonding), for vegetable tannins for instance. However, the binding of cationic chromium complexes was somewhat lowered, indicating impaired reactivity of the carboxyl groups The function of the cationic protein groups was unaltered Since periodate solutions of 0 25 molar strength are able to oxidize carbohydrates during the experimental conditions applied, *it* appears that the small amount of sugar present in thoroughly *limed* collagen does not contribute to its stabilization. Solutions of greater strength, such as 0.5 molar $NaIO_4$, have a drastic effect on collagen. About 80-90 per cent of the bovine skin collagen is solubilized. The *shrinkage temperature is* lowered from 60-65° C. to 35-40° C. Moreco

development of chick embryos. Up to the twelfth day there is a decrease in metachromasia both in cornea and sclera; the twelfth and thirteenth day metachromasia is practically absent. From the fourteenth day onward metachromasia in the cornea increased gradually, on the last – twenty-first – day of embryonic life the maximum intensity is reached. The sclera stains orthochromatically from the twelfth day onwards. The cornea becomes transparent at about the same time. from the fourteenth to the twenty-first day the amount of transmitted light increases from 60 to 100 per cent.

Smits has made hexosamine determinations in corneas and scleras of bovine foetuses of increasing age. Up to a total length of 25 cm. both tissues show a decrease in hexosamine; from 25 cm. onward the hexosamine content of the cornea increases again, while the content of the sclera decreases further After birth the hexosamine-HCl content of cornea and sclera are 2.48 per cent and 0 58 per cent respectively.

(2) Among non-histologists there is a tendency to call thin collagenous fibrils 'reticulin' As reticulin is defined as connective tissue fibrils showing argyrophilia, it is not justifiable to identify the aforementioned fibrils with reticulin, because the mucoid-containing tissues cannot be impregnated with silver So fibril thinness in itself is not sufficient to cause argyrophilia, therefore thin fibrils, which can be impregnated with silver (reticulin) must be distinguished from thin fibrils lacking this characteristic.

Fibrils of both types were examined with the electron microscope after impregnation with silver. Care was taken that possible relations between fibrils and interfibrillar substance were maintaine a certain extent, therefore a simple mechanical fragmentati technique was applied. Cornea and cartilage were chosen as tissu rich in mucoid, spleen and kidney as organs containing much ret culin. Embryonic skins of increasing age were studied also, as n developing skin there is a transition of reticulin into collagen.

The results can be summarized as follows: whenever argyrophilia is present – recognizable at the electron microscope level by big silver grains – a membrane-like matrix is visible between the fibrils (Fig 5); this applies to the reticulin of spleen and kidney, in young embryonic skin the matrix can be shown also, but the membranous character is less obvious. The fibrils of cornea and cartilage are covered with very small silver grains – corresponding with th

which has a much higher carbohydrate content, is translucent in thin layers, but not transparent. If one uses hydraulic pressure on the cornea it is possible to extract a great deal of polysaccharide of two different types — one with electrophoretic mobility of a non-sulphated polysaccharide and the other of a sulphated polysaccharide There is metachromasia of the substantia propria, but Descemet's membrane shows no metachromasia The question is this: is Descemet's membrane collagen or not? Dr Gross said yes; it contains 10 per cent hydroxyproline and twice that amount of glycine.

[illegible] of [illegible] tio [illegible] would render it opaque Apparently the changes in refractive index of the fibrils by tension are sufficient to make the cornea opaque.

Dr. Fitton Jackson said that presumably the pressure closed up the 'lattice'. The refractive index of normal cornea is 1 52 and when this is altered by hydration or dehydration then the optical properties of the cornea impair sight owing to a change in the refraction index between the fibres and interfibrillar materials

Dr Snellman mentioned work done some years ago by Engstrom and Caspersson which showed that the different layers of the cornea had different refractive indices

Dr Gillman pointed out that the lens seemed to have received much less attention than the cornea In certain metabolic disturbances the opacity of the two may run together. He wondered, in particular, whether any studies were available at the electron microscope level, on extracted or sectioned material, on alterations in the morphology of the fibres in the cornea or the lens in experimental diabetes In alloxan diabetes development of corneal and lens opacity seem to be closely related to carbohydrate metabolism The other point was that as in the arteries, so too in the lens and in the cornea, alterations in the metabolism of the individual could produce tendencies towards the binding of certain substances, for example in the arteries, i e lipids and minerals Thus calcium, iron and lipid may be more easily bound on corneal fibrils in the presence of particular types of systemic metabolic disturbances affecting corneal metabolism

Dr Meyer said he did not know of any correlation between corneal and lens carbohydrate moieties in both the cornea and the lens opacity apart from infections as in vitamin deficiency states He did not know that the two went hand in hand in diabetes.

brown colour in the light-microscope (Fig. 4); the presence of matrix as mentioned above could not be shown. Apparently th matrix of reticulin has a far greater mechanical stability than mucoi which can be washed off rather easily.

Instructive are the pictures one can observe in the same specimer of spleen c
able by tl
substance
shown by the bundling of the fibrils and the smallness of the silver

between fibrils and matrix.

GROUP DISCUSSION

Dr. Gross stated that Dr. Marie Jakus at the Retina-Foundation in
similar
Ång-
n and
wn a
hexa-
gonal type, 1000 to 1500 Angström apart. Cornea is the only known

len Hooff had exam-
den Hooff said yes,
transparency in the
material earlier than eight days Dr. Fitton Jackson said that by using polarized light, laminated layers of fibres can be seen in ten- or eleven-day-old embryos

Dr. Neuberger asked how the constituents of the cornea compared

gures but the
5 per cent
mucopolysaccharide and 30 per cent non-collagenous protein. Sclera (dry weight) contained 80 per cent collagen, 1 per cent mucopolysaccharide and about 10 per cent non-collagenous protein

Dr. Meyer said this problem of transparency of the cornea was an intriguing one. *Maurice tried to explain it* on the basis of a diffraction grating formed by the different fibril layers On drying the transparency disappeared and it became translucent. In this respect, hyaline cartilage,

which has a much higher carbohydrate content, is translucent in thin layers, but not transparent If one uses hydraulic pressure on the cornea it is possible to extract a great deal of polysaccharide of two different types —one with electrophoretic mobility of a non-sulphated polysaccharide and the other of a sulphated polysaccharide. There is metachromasia of the substantia propria, but Descemet's membrane shows no metachromasia The question is thus is Descemet's membrane collagen or not? DR GROSS said yes; it contains 10 per cent hydroxyproline and twice that amount of glycine

DR MEYER said the grating theory did not entirely explain the opacity of the cornea. It must have something to do with alteration in the hydration of the tissue components. It would also be altered if a new type of collagen grew in, as in leucoma

DR VAN DEN HOOFF agreed that the transparency of the cornea was only within very narrow limits of hydration, even pressure on the cornea would render it opaque. Apparently the changes in refractive index of the fibrils by tension are sufficient to make the cornea opaque

DR FITTON JACKSON said that presumably the pressure closed up the 'lattice' The refractive index of normal cornea is 1 52 and when this is altered by hydration or dehydration then the optical properties of the cornea impair sight owing to a change in the refraction index between the fibres and interfibrillar materials.

DR SNELLMAN mentioned work done some years ago by Engstrom and Caspersson which showed that the different layers of the cornea had different refractive indices.

DR GILLMAN pointed out that the lens seemed to have received much less attention than the cornea. In certain metabolic disturbances the opacity of the two may run together He wondered, in particular, whether any studies were available at the electron microscope level, on extracted or sectioned material, on alterations in the morphology of the fibres in the cornea or the lens in experimental diabetes. In alloxan diabetes development of corneal and lens opacity seem to be closely related to carbohydrate metabolism The other point was that as in the arteries, so too in the lens and in the cornea, alterations in the metabolism of the individual could produce tendencies towards the binding of certain substances, for example in the arteries, i e lipids and minerals Thus calcium, iron and lipid may be more easily bound on corneal fibrils in the presence of particular types of systemic metabolic disturbances affecting corneal metabolism

DR MEYER said he did not know of any correlation between corneal and lens carbohydrate moieties in both the cornea and the lens opacity apart from infections as in vitamin deficiency states. He did not know that the two went hand in hand in diabetes.

DR GILLMAN said they can, under certain experimental
Opacity of the cornea is not always a function of infection. I
in riboflavine deficiency, vascularization of the cornea m
and lead to opacity unassociated with infection.

DR. MEYER presumed that this was due to the productior
collagen fibrils during the vascularization process.

DR GILLMAN said his main point was that the morpholo
try of the fibres of the lens and cornea did not seem to ha
attention they merited either by the light or the electron

DR. SCHWARZ pointed out that the first function of
transparency. A change in fibril or a change in ceme
would alter the refraction. The second function was the r
permanent curvature for the whole life of the organism
can be corrected surgically by an incision at the cornea
which alters this curvature.

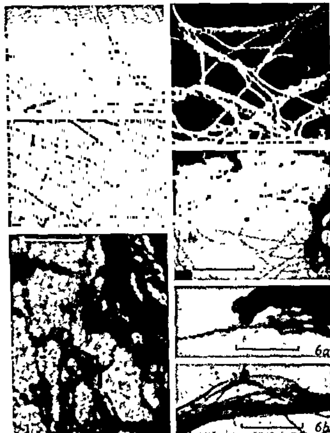


FIG 1
Replica of bull's sclera × 15 000

FIG 2
Replica of bull's cornea × 15,000

FIG 3
Fibrils of bull's cornea × 20 000

FIG 4
Fibrils of bull's cornea impregnated with silver × 17,000

FIG 5
Reticulin from kidney of bovine foetus of 60 cm, impregnated with silver × 17,000

FIG 6
Fibrillar material from the spleen of a new-born pig, the pictures are of the same specimen (a) reticulin, (b) collagenous material × 17,000



WHAT IS RETICULIN?

A. H. T. Robb-Smith

Reticulin fibres were first described by Kupffer in 1876 and eight years later Mall (1888) claimed that it was distinct from collagen;

characters, as revealed by the light microscope. It would seem

right angles; it stains faintly with acid fuchsin (van Gieson's stain), accepts the acid aniline dyes in the Mallory or Masson Trichrome method, and appears black in both toned and untoned sections impregnated with silver by the Maresch-Bielchowsky method.

There has been some disagreement as to its reaction with the perio… technique used, but … stains strongly, whe… ll (Robb-Smith, 1952), with the sulphation metachromasia reaction of Kramer and Windrum (1953-54) reticulin is metachromatic, while collagen is orthochromatic.

Native collagen and reticulin fibres resist tryptic digestion but undergo dissolution when incubated with clostridial collagenases (Robb-Smith, 1945, 1953; Dresner and Schubert, 1955).

In the identification of reticulin it is not sufficient to depend on one character alone, and there is no doubt that in the strict sense in which it is proposed to use the term here it would be quite wrong to assume that all fibres which appear black in the light microscope when impregnated with silver by one of the modifications of the Maresch-Bielchowsky method are reticulin. These silver impregnation methods are not histochemical tests and for them to be meaningful, it is necessary for both the solutions and material to be controlled

critically as it is perfectly easy for any of the tissue elements in a preparation to appear black, brown or yellow if the conditions are so adjusted.

In mature connective tissue, reticulin often appears to merge with collagen fibres but the angular branching is very characteristic and predominantly it *is seen in relation to the basement membranes* between connective tissue and epithelium and around muscle and nerve fibres, etc.

However, it has been shown (Lillie, 1952) that the staining reactions of basement membranes are not uniform, suggesting variability in their constituents and although in general it would be correct to say that reticulin is a component of mammalian basement membranes, there are exceptions to this. For example, the basement membrane of the normal human renal glomerules contains no reticulin and its staining reactions are markedly different (Rinehart *et al.*, 1953), while electron microscopic studies (Benedetti and Tivibelli (1954), Pease (1955) have failed to reveal the presence of collagen-type fibrils.

This is of some importance as Cruickshank and Hill (1953), using the fluorescent antibody technique of Coons and Kaplan (1950) showed that anti-glomerulus serum 'stained' both renal glomerular basement membrane and reticulin, but not collagen fibres and they quite rightly stated that their experiments showed the presence of a common antigen in these two tissue elements, but this work has been misinterpreted as a specific method for identifying reticulin.

In embryonic and regenerating connective tissue, the angular branching reticulin fibres are seen quite early (in the human foetus by 38 days, in healing wounds in 6-8 days) but another type of argyrophil fibre is also present which has often been called reticulin, but I *believe should be distinguished from it*; there are fine wavy

you like, collagen fibres, and clearly if they are to be called reticulin then a different name must be adopted for the angular branching argyrophil fibres of basement membranes and mature connective tissue.

There is a further variety of argyrophil fibres which has been very little studied and the *only site in which I am confident* of their existence is the ovarian stroma (Robb-Smith, 1952). They are pre-

sent in association with reticulin and I have only been able to display

thus, as one has no idea what other changes have been induced in the tissues by this technique.

Bembridge (1952) has described collagenase-resistant trypsin digestible fibres in the ciliary region of the vitreous and Martin

Since the days of Nageotte and Huzella, it has been shown that

well be of value in elucidating the factors that determine the argyrophil reaction, yet because of the relative non-specificity of these reactions under abnormal conditions, I do not believe attempts should be made to draw very close analogies between results ob-

(1954).

In my department we have been interested in reticulin for a good long time and in an endeavour to characterize it more precisely, it was necessary to find a tissue rich in reticulin and poor in collagen, and after a survey of a wide range of tissues, we adopted the subcortical tissue of the kidney as its fibrillary portion consisted almost exclusively of reticulin, as I have defined it here, and it was comparatively easy to dissect out the small amounts of collagen around the blood vessels.

Using material of this type, Kramer and Little (1952) studied it

confirmed these findings X-ray diffraction photographs (Little and Windrum, 1954) showed a pattern quite characteristic of collagen, but in addition to the collagen bands other rings were seen at

critically as it is perfectly easy for any of the tissue elements in a preparation to appear black, brown or yellow if the conditions are so adjusted.

In mature connective tissue, reticulin often appears to merge with collagen fibres but the angular branching is very characteristic and predominantly it is seen in relation to the basement membranes between connective tissue and epithelium and around muscle and nerve fibres, etc.

However, it has been shown (Lillie, 1952) that the staining reactions of basement membranes are not uniform, suggesting variability in their constituents and although in general it would be correct to say that reticulin is a component of mammalian basement membranes, there are exceptions to this. For example, the basement membrane of the normal human renal glomerules contains no reticulin and its staining reactions are markedly different (Rinehart *et al.*, 1953), while electron microscopic studies (Benedetti and Tivibelli (1954), Pease (1955) have failed to reveal the presence of collagen-type fibrils.

This is of some importance as Cruickshank and Hill (1953), using the fluorescent antibody technique of Coons and Kaplan (1950) showed that anti-glomerulus serum 'stained' both renal glomerular basement membrane and reticulin, but not collagen fibres and they quite rightly stated that their experiments showed the presence of a common antigen in these two tissue elements, but this work has been misinterpreted as a specific method for identifying reticulin.

In embryonic and regenerating connective tissue, the angular branching reticulin fibres are seen quite early (in the human foetus by 38 days, in healing wounds in 6-8 days) but another type of argyrophil fibre is also present which has often been called reticulin, but I believe should be distinguished from it, there are fine wavy fibres which do not branch and merge very closely with the developing collagen fibres and indeed appear to be replaced by the collagen fibres. I think these should be regarded as immature, argyrophil, if you like, collagen fibres, and clearly if they are to be called reticulin then a different name must be adopted for the angular branching argyrophil fibres of basement membranes and mature connective tissue.

There is a further variety of argyrophil fibres which has been very little studied and the only site in which I am confident of their existence is the ovarian stroma (Robb-Smith, 1952). They are pre-

sent in association with reticulin and I have only been able to display [illegible]

Bembridge (1952) has described collagenase-resistant trypsin digestible fibres in the ciliary region of the vitreous and Martin [illegible]

[illegible]

reticulin if the term is to be used for the biological structural element [illegible]

reactions under abnormal conditions, I do not believe attempts should be made to draw very close analogies between results obtained in this way and the structure and nature of reticulin as I have defined it, and for the same reasons, I do think too much significance can be attached to the interesting experiments of Irving and Tomlin (1954)

In my department we have been interested in reticulin for a good long time and in an endeavour to characterize it more precisely, it was necessary to find a tissue rich in reticulin and poor in collagen, and after a survey of a wide range of tissues, we adopted the subcortical tissue of the kidney as its fibrillary portion consisted almost exclusively of reticulin, as I have defined it here, and it was comparatively easy to dissect out the small amounts of collagen around the blood vessels.

Using material of this type, Kramer and Little (1952) studied it with the [illegible] work [illegible] 640 Å [illegible] confirmed these findings. X-ray diffraction photographs (Little and Windrum, 1954) showed a pattern quite characteristic of collagen, but in addition to the collagen bands other rings were seen at

2 ... hasting
... material ...
position is very similar to that of collagen ... it to the extent of 4.2 per cent non-hexosamine, the sugars identified being galactose, mannose and fucose; no uronic acid or sulphate ester was detected. In addition, it contained 10.9 per cent bound fatty acids of which about 95 per cent was myristic acid, the rest palmitic. Thus, it appears that reticulin as I have defined it, from human kidney, is a lipo-glyco-protein in which the amino acid constituents are very similar to those in collagen.

It seemed desirable to try and ascertain what relationship, if any, reticulin had to the various soluble collagens that have been described; any isolation process appeared unsatisfactory as it would be difficult to relate the isolated fragments with histologically defined structures, and so an attempt was made to expose tissues to various buffer solutions and then examine them histologically.

The tissue used was human kidney, obtained six hours after death from a child, who had died as a result of an accident and showed no pathological lesions in the viscera. Portions of kidney, unfixed, were cut on a freezing microtome and slices about 100μ thick were obtained. Some of these were immediately fixed in 4 per cent saline formaldehyde as controls (Figs. 1 and 2), others were placed in large volumes of the buffer solutions and gently agitated while being kept in a refrigerator at approximately 2° C.; at the end of the period of ex... they were fixed in 4 per cent saline formaldehyde. All the ... and sections were cut at 7μ and ... xylin and ... plasia of Kramer and Windrum, ... Schiff and Robb-Smith's silver impregnation method ... r each staining method all the sections were treated simultaneously for identical times in order to avoid uncontrolled variations in staining.

The solutions to which the unfixed sections of kidney tissue were exposed were as follows:

(1) 0.2 M phosphate buffer pH 8.8 for 12 hours; (2) 0-1 N acetic pH 2.73 for 12 hours; (3) 0.005 N sodium hydroxide pH 12

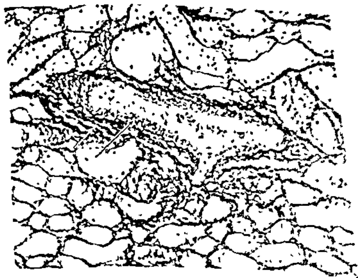


Fig. 1



Fig. 2



FIG 3



FIG 4

Fig 3 Phosphate buffer pH 8 8 for 12 hours at 2° C followed by 0 1 M citrate buffer pH 3 8 for 12 hours at 2° C
Fig 3 × 420, Fig 4 × 1,400

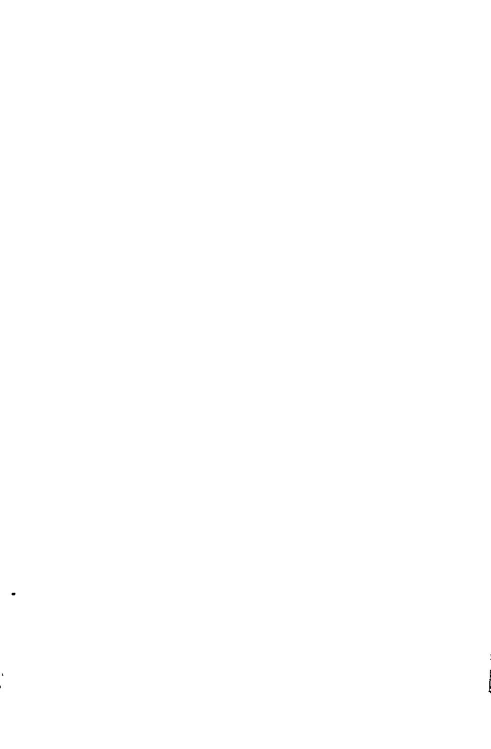


FIG 5



FIG 6

C. Phosphate buffer pH 8 8 for 12 hours at 2° C followed by acetic acid pH 2 7 for 12 hours at 2° C.
Fig 5 × 420, Fig 6 × 1,400



for 12 hours, (4) 0.1 *M* citrate buffer pH 3.8 for 12 hours (Figs. 3 and 4); (5) phosphate buffer for 12 hours followed by acetic acid for 12 hours (Figs. 5 and 6); (6) phosphate buffer 12 hours, followed by alkali for 12 hours; (7) phosphate buffer for 12 hours followed by citrate buffer for 12 hours.

It is not proposed to describe in detail the histological changes that have been observed, but in essence it was found that following the exposure of the tissue to their various buffers, there was no tinctorial or structural alteration of the reticulin as observed with the light microscope but that there were marked alterations in the staining reaction of the collagen and ground substance in all the preparations, except those exposed to phosphate buffer alone. The most striking diminution of collagen staining was observed in the sections treated with phosphate buffer followed by citrate buffer and phosphate buffer followed by acetic acid.

A similar experiment was set up in which the material used was granulation tissue from a healing wound which was rich in the fine wavy non-branching argyrophil fibres which I have mentioned on page 178. In these experiments it was found that the buffer solutions had no effect on argyrophil reticulin fibres, but there was disappearance and loss of silver staining of the wavy argyrophil fibres and again this was most marked in the sections treated with phosphate and citrate buffers and phosphate buffer and acetic acid.

The methods of extraction used correspond closely to those adopted for the extraction of soluble collagens and although no attempt was made to demonstrate a soluble collagen in the buffers after the tissue had been in them, yet the histological evidence, for what it was worth, suggested that collagen had been extracted from the tissue and also that there had been extraction of unmature argyrophil collagen fibres, but no change could be detected with regard to reticulin.

There have been few, if any, integrated morphological and chemical studies of connective tissue after extraction of chemically identifiable fractions, but a relevant study is that of Tustanowski *et al.* (1954) on collastromine (there may well have been more recent papers on this subject which have not been read). Tustanowski *et al* showed that after repeated extraction of rat skin with citrate buffer pH 4.0 and final extraction with water at 50° C. for an hour and a half, the residue consisted of fibres which were argyrophil, but did

tissue components such as elastica, and detailed chemical analysis of the residues after extraction of collagen from connective tissue have not, as yet, been made, nevertheless, it seems very probable that reticulin accounts for some of the properties of collastromine.

In this paper, I have tried to show the difficulties in identifying reticulin and the progress that has been made up to date. Reticulin as defined here does not appear to be a precursor of the mature collagen fibre, but a biological fibre of the collagen family, of considerable stability, which is unlikely to form the structural basis of the collagen fibre. It is desirable to distinguish reticulin as defined here from other argyrophil fibres which in the past have been called 'reticulin' of which the most important example is the immature argyrophil collagen fibre to be found in areas of fibrillogenesis whether embryonic or reparative.

GROUP DISCUSSION

Dr Snellman said the silver staining might be due to aldehyde groups derived from any acetal.

Dr D S. Jackson said that the major proportion of the fatty acid is myristic, and palmitic and stearic might be impurities

Dr Robb-Smith replied that at first he also thought it was an impurity but, as it was found in constant amounts, he was forced to the conclusion that it was not

Dr Consden stated that the work of Eastoe showed conclusively that myristic acid was released only after hydrolysis Combination of myristic acid with protein might account partially for the resistance of reticulin to different treatments Dr Consden was interested in the autoclavibility of reticulin in order to remove it from tissue residues. He had found, using kidney reticulin, that about 85 per cent of hydroxyproline could be extracted after autoclaving for nine hours However, a small amount continued to come out if autoclaving was prolonged

Dr Meyer said sialic acid was a strong reducing agent and easily split off It occurred in granulocytes, peptides and carbohydrates.

Dr Reed asked Dr Robb-Smith whether there was an increase of elastic tissue staining after treatment with phosphate buffer at pH 8 8 Dr. Robb-Smith replied that there was no increase in elastic staining with orcein as far as the blood vessels were concerned; but the tissue had been exposed to the buffers for only 12 hours at 2° C.

Dr Partridge asked Dr. Consden whether his residue after autoclaving had been examined for polysaccharide Dr. Consden replied that un-

fortunately 50 per cent of inorganic material was present so it could not be analysed accurately. It contained very little hydroxyproline after [illegible]

number of processes and disappear, but once it was laid down as basement membrane it probably would not change to normal mature collagen. Reticulin was not a half-way house to mature collagen fibres but a 'branch line'.

SOME NEW ASPECTS OF THE STABILITY AND REACTIVITY OF COLLAGENS

K. H. Gustavson

INTRODUCTION

It should be noted at the outset, that my contribution will be restricted firstly to a review of results, partly unpublished, which have been obtained on investigating the nature of the valency forces responsible for the stabilization of the polypeptide chains present in the protofibrils and the filaments of collagen. Thus, reactions and forces involving distances less than 50–100 Ångström units are concerned (Bear, 1952). Information on the type of side chains and links in the polypeptide backbone forming the sites for these valency forces will hence be a principal issue. A comprehensive review has recently been given by Gustavson (1956a).

Secondly, some problems, which might be involved in the altera-

[illegible]

cross-linking and tanning processes as an explanation of the alteration of collagen in the organism under certain conditions.

TYPE OF CROSS-LINKS

It is generally considered that the cohesive forces between the elementary units of the collagen fibril are of three types

(1) *ionic valence*, mainly in the form of salt-like cross-link between oppositely charged, long side chains, as those between the glutamic acid and lysine residues, the commonly cited example (Speakman and Hirst, 1931, 1932, Lloyd *et al*, 1933).

(2) *non-ionic* cross-links, the most important one being the hydrogen-bond, e.g the short link formed by the keto-imide groups on adjacent protein chains (Pauling and Niemann, 1939). The presence

fortunately 50 per cent of inorganic material was present so it could not be analysed accurately. It contained very little hydroxyproline after [illegible]

cular stabilizing bridges. It is of the order of some 60° C., whereas the degree of stabilization by the salt-like links is of the order of 12-14° C. on the *Ts* scale only. Thus, such a large amount as at [illegible]

expected to be rather slight in view of the effect of the great D.E. constant of the aqueous solutions on the fibres.

At this point it is appropriate to note that the effective valency range of the sites of the ionic link is of the order of 50-100 Ångström units, while that of the dipolar forces responsible for the hydrogen bond and for the co-ordinate bonds generally is a few Ångström units only (Bear, 1952). Then, the latter forces cannot be operative between larger units of the collagen (skin) structure, such as fibres, whereas electrovalent links may be able to extend their force across interfibrillar spaces of some 50-60 Ångstrom units. They may accordingly influence the mechanical strength of the fibres, their cohesion. It is noteworthy that in the light of the available experimental material, the *hydrothermal stability* of collagen appears to be governed by the short links between polypeptide chains (helices), and thus to be a function of the number and the strength of the hydrogen bonds present (Gustavson, 1948), whereas the *tensile strength* of a *single fibre bundle* apparently is mainly determined by surface forces of weakly polar nature, influencing the frictional forces between the fibres This view receives some support by the [illegible]

Mao and Roddy, 1950, Roddy, 1952).

TYPE OF HYDROGEN BONDS

As to the number of hydrogen bonds of the ordinary type on the —CO.NH— groups, generally conceived to be the principal ones (5) – and also believed by some to be the only type existing – it appears that presently one systematic set of hydrogen bonds is [illegible]

(3) Van der Waals forces, and similar links formed by other weakly polar groups.

To which extent these various intermolecular forces, or types of co-ordinate bonds, contribute to the stabilization of the fibre structure is the second cardinal question. *Using the degree of the hydrothermal stability of collagen, i.e. the value of the temperature of the instantaneous shrinkage of the fibre in water, the Ts, some indication, or an approximation of the relative importance of the ionic and the non-ionic protein groups in the cross-linking of collagen is obtainable* (Gustavson, 1942a, b; 1946a).

It appeared to the present author that by ascertaining the effect of the complete inactivation of the ionic groups of collagen, the acid and base binding ones, on the shrinkage temperature by an agent which is irreversibly fixed by collagen and which does not swell the [illegible] sulphonic acids (Bergmann *et al.*, [illegible] en [illegible] nt to be used for the severance of the salt-like bridges must be irreversibly fixed by collag[illegible] of the collagen secondary [illegible] links take place, wh[illegible] problem. *By saturation of collagen with β-naphthalene sulphonic* acid on equilibration with solutions of pH 1.5, the shrinkage temperature *of calf-skin* collagen is lowered 12-14° C. (Gustavson, 1942). This impaired stability is generally conceived to be the result of eliminating the ionic forces between the polypeptide chains. Similar results have been obtained by Jackson (1953, 1954) on tendon collagen. By the treatment of the skin in concentrated solutions of some strongly lyotropic agent, such as calcium thiocyanate of 2-molar strength, the fibre will shrink in the treatment, even at temperatures of 10° C. or lower (Kuntzel, 1937). There are indications favouring the view that the lyotropic shrinkage is intimately [illegible] cross-links

particularly that of fish-skin collagen, was too fragmentary at that time, no definite explanation of the greater availability of non-compensated keto-imide groups indicated in the teleostean collagen was possible. The complete analytical data of the amino acid content of collagen of bovine skin (Bowes and Kenten, 1948) and fish skins, which were later forthcoming (Neuman, 1949; Neuman and Logan, 1950), showed the main difference between collagen of mammals and teleosts to be the low content of hypro (9 per cent) of fish-skin collagen compared to 14 per cent for bovine collagen, apart from

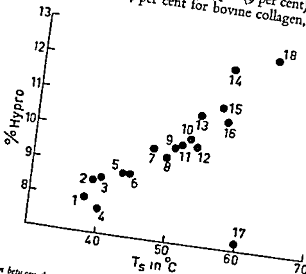


Fig. 1

The relation between the Ts (in ° C) and the hydroxyproline content of collagens of the skin of various fishes in the native state

The numbers on the dots represent

(1) Alaska pollak, *Theragra*
(2) Atka mackerel, *Pleurogrammus*
(3) Flathead flounder, *Hippoglossoides*
(4) Codfish, *Gadus*
(5) Flounder, *Tanakius*
(6) Small-mouthed sole, *Limanda*
(7) A kind of flounder, *Lepidosetta*
(8) Japanese halibut, *Paralichthys*
(9) Japanese mackerel, *Scomber*
(10) Yellowtail, *Seriola*
(11) Jack mackerel, *Trachurus*
(12) Fresh water eel, *Anguilla*
(13) Great blue shark, *Prionace*
(14) Wild goldfish, *Carassius*
(15) Carp, *Cyprinus*
(16) Fin whale *Balaenoptera*
(17) *Horny fibre of shark*
(18) *Cattle*

Note The only specimen not falling into line is No 17. The chief protein of the shark fins is elastoidin which in many respects behaves extraordinarily, compared to the collagen of skin, e.g. by showing rubber-like behaviour after thermal shrinkage (Fauré-Fremiet, 1937, Fauré-Fremiet and Woelfflin, 1936, Champetier and Fauré-Fremiet, 1937). Elastoidin con-, hur-bridges probably. It shows the Ewald reaction of extensibility also which would consistent with the presence of covalent cross-links (Fauré-Fremiet, 1937). Cf Damodoran, , *et al* (1956) *Biochem J*, 62, 621

three-fold screw axis with the chain winding round each other to form a coiled-coil (Crick, 1956; Cowen *et al.*, 1955; Ramachandran, 1956, Ramachandran and Kartha, 1954, 1955; Reed *et al.*, 1956). The sequence: gly-pro-hypro can be accommodated in this model (Kroner *et al.*, 1953; Schroeder *et al.*, 1954). In view of this recent development, according to which the molecule of collagen consists of a unit of three helices, it should be noted that the term *intermolecular* or *interchain* cross-link, as used in earlier publications as well as in the present paper, should be identified with the link connecting two molecules of the trihelical unit type, while the *intramolecular* cross-link should apply to bonds between the three polypeptide chains in the unit.

The presence of strong hydrogen bonds between the hydroxy group of the hypro residue and some other link or group on an adjacent polypeptide chain, probably the keto-oxygen of the keto-
the results from
·55a). The data
molecular bond

$-OH \ldots OC\langle^{NH-}$ can readily be accommodated in the trihelical model, and actually that such a cross-link is a logical consequence of this particular structure. This type of link was independently suggested by Huggins (1954) in his latest model of collagen, based on data from X-ray diffraction, and by the present author (1954a, b) from his investigations of the chemical reactivity and the hydrothermal stability of various types of collagen.

HYDROTHERMAL STABILITY AND HYDROXYPROLINE CONTENT

In studies of the hydrothermal stability of collagen of skins of mammals and fishes (mainly bovine skin and skin of cod fish, respectively), measured by the temperature of instantaneous shrinkage, *Ts*, it was found that the *Ts* of native skins of mammals generally falls in the range 60–70° C., while those of fish skin are much lower, for skins of cold-water fish 35–45° and for skins of warm-water fish 45–55° C. (Gustavson, 1953; Takahashi and Yokoyama, 1954). It was pointed out in the early papers (Gustavson, 1942b, c; 1949; 1950b) that the hydrogen bond type of cross-link appeared to be less developed in the collagen of fish skin than in that of bovine skin. Since information on the amino acid composition of these collagens,

particularly that of fish-skin collagen, was too fragmentary at that time, no definite explanation of the greater availability of non-compensated keto-imide groups indicated in the teleostean collagen was possible. The complete analytical data of the amino acid content of collagen of bovine skin (Bowes and Kenten, 1948) and fish skins, which were later forthcoming (Neuman, 1949; Neuman and Logan, 1950), showed the main difference between collagen of mammals and teleosts to be the low content of hypro (9 per cent) of fish-skin collagen compared to 14 per cent for bovine collagen, apart from

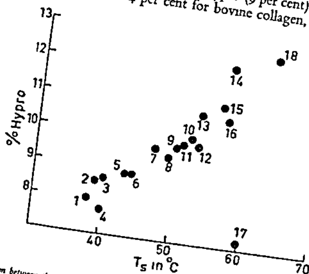


FIG 1

The relation between the Ts (in ° C) and the hydroxyproline content of collagens of the skin of various fishes in the native state

The numbers on the dots represent

(1) Alaska pollak, *Theragra*
(2) Atka mackerel, *Pleurogrammus*
(3) Flathead flounder, *Hippoglossoides*
(4) Codfish, *Gadus*
(5) Flounder, *Tanakius*
(6) Small-mouthed sole, *Limanda*
(7) A kind of flounder
(8)
(10) Yellowtail, *Seriola*
(11) Jack mackerel, *Trachurus*
(12) Fresh water eel, *Anguilla*
(13) Great blue shark
(14)

minor divergences in amino acids present in small amounts, such as serine, threonine and methionine. Hence the possibility of the hydroxy group of the hypro residue being involved in the intermolecular linking of collagen was obvious. The original view of the less-developed intermolecular hydrogen bonding on the peptide links of fish collagen had thus to be modified with due attention to the possible function of the hydroxy group in the interchain linking (Gustavson, 1953).

[illegible]

the hypro have been estimated by an indirect method colorimetrically, a set of figures for the *directly* estimated contents of hypro and proline in the three types of collagen are given in Table I in gm.

TABLE I

CHARACTERISTIC PROPERTIES AND CONTENTS OF PROLINES OF COLLAGENS (GUSTAVSON, 1954a, 1955a)

No	Type of skin	Ts in ° C	Per cent total nitrogen	Acid binding capacity in mmol HCl/gm collagen	Per cent Proline	Per cent Hypro	Per cent Prolines
1	Cod fish	40	18.3	0.92	10.8	6.0	16.8
2	Pike	55	18.4	0.93	13.3	7.9	21.2
3	Bovine (calf skin)	66	18.3	0.90	14.1	12.7	26.8

[illegible] collagen. The skin of cod fish represents the [illegible] of warm- [illegible] type of collagen.

Among collagen-like proteins in organism of invertebrates, the protein of the cuticle of the earthworm (*Allolobophora longa*) is perhaps best known in structural and chemical respects; the secreted type of collagen having mainly been studied from the cytological point of view. The cuticle protein which shows the wide-angle X-ray diagram characteristic of collagen (Reed and Rudall, 1948),

shrinks in water at 40° C., yielding a solution which does *not* gel on cooling.

The total nitrogen content of the dry cuticle is reported to be 14.5 per cent only. The protein contained 15 per cent of its total nitrogen in the form of hypro and only 3 per cent of proline, according to analyses by Mr. Singleton in Professor Astbury's laboratory which were kindly communicated by Professor Astbury, and reported earlier (Gustavson, 1955b). Hence in this instance, in spite of the great amount of hypro, the hydrothermal stability is low. However, it should be recalled in this connection that stretched gelatin gel, which gives the wide angle diffraction diagram of collagen, has its melting point in the range of 35-40° C. in spite of its content of hypro being the same as that of bovine collagen, with *Ts* of the order of 65° C. According to Reed and Rudall (1948), the earthworm protein is non-striated. In view of the non-gelling of the solution of the melted cuticle protein and with due consideration of the aforementioned facts regarding gelatin and non-striation, it seems doubtful whether the earthworm protein should be cons[illegible] to be a member of the collagen family.

Watson and Sm[illegible] the amino acids [illegible] 14.6 per cent nitr[illegible] about 80 per cent of collagen. [illegible] contained The contents of glycine and non-polar amino acids were practically identical with those of bovine collagen. The proline figure was only 1.2 per cent of the total protein nitrogen, while the hypro was present in such a large amount as 12 6 per cent Thus, earthworm cuticle contains a higher proportion of hydroxyprolyl residues than any collagen yet analysed. Also the aliphatic hydroxy-amino acids are higher in the cuticle protein than in the bovine collagen, whereas the contents of lysine and arginine are appreciably lower. The protein sulphur is appreciably lower than that of bovine collagen (<0.1 per cent S). Evidently, additional information on the fine-structural details is desirable before any theorization on the implication of the hypro content — hydrothermal stability is justified in this particular instance.

In this connection it should be noted that the reptile skins which are commercially used, such as the skin of the alligator and the crocodile, shrink at 60-63° C. The lizard skins of commerce have *Ts* of 62-65° C. The degree of structural stability of the snake skins of importance in leather manufacture is of the same order. It is in-

teresting to note that the skin of the Australian lung-fish belongs to the mammalian type of skin as far as hydrothermal stability is concerned (*Ts*: 65–67° C.).

Obviously, the data in Fig. 1 and Table I are no evidence in themselves for the view that the hypro residue is directly involved in the stabilization of collagen; for example by forming a site for an interchain hydrogen bond. The effect of the irregularity of the polypeptide chains imposed by the prolines may be indirect and even fortuitous.

However, if collagen is mainly stabilized by interchain hydrogen bonds on oppositely located keto-imide groups of adjacent chains, the content of pyrrolidine residues should be a factor of importance. The residues of the prolines which are built into the polypeptide chains with the formation of –CO.N– links introduce a regular interruption of the normal –CO.NH– links, formed by the re mainder of the amino-acid residues. Accordingly, sites for the com pensation of adjacent –CO– groups of the keto-imide links a withdrawn, which implies fewer stabilizing cross-links and co sequently a lowered degree of stability. Then, mammalian collag should be less stable to heat than fish collagen. However, the reve is true.

INTERCHAIN HYDROGEN BOND BETWEEN HYDROXY AND KETO-IMI GROUPS

On the other hand, supposing that the hydroxy groups o residues of the hydroxyamino acids may enter into interchain h gen bonding with some other groups, for example, the carbo the carbonyl of the –CO.NH– linkage, the stability of the str would be expected to increase with the content of hydroxy as is actually found.

A satisfactory explanation of the correlation of the hydr line content of collagens and their *Ts*, and indications of the b link formed, have been arrived at by investigations of the b of exhaustively acetylated bovine collagen (Gustavson, 1954 Such a collagen with all its amino-groups (0.39 mmol./gm. and 80 per cent of its hydroxy groups inactivated (1.39 m collagen) was obtained by acetylating hide powder and according to the method of Green *et al.* (1953). Compa periments with N-acetylated collagen were included. T

thermal stability of collagen was not affected by its N-acetylation, while the N- and O-acetylation, that is, the blocking of the hydroxy- and amino-groups, lowered the *Ts* from 64–66° C. to 40–44° C. (Gustavson, 1954a, b, 1955a). This finding indicates the rupture of *interchain* cross-links by the O-acetylation.

Assuming that the aliphatic hydroxy groups are preferentially acetylated, at least 0.8 mmol. of hydroxyproline per gm. collagen should have reacted. Hence, out of 1.07 mmol. hydroxyproline residues of the original collagen, three out of four hydroxy groups have been blocked. In this connection, it is of interest to note that *Ts* for the O-acetylated bovine collagen coincides with that of the skins of most cold-water fishes, suggesting that the hydroxy group, resisting acetylation, forms a stronger bond than the rest of the hydroxy groups, and further that this particular group is mainly responsible for the stabilization of the teleost collagen.

From investigations of the effect of the inactivation of the hydroxy groups of collagen on its reactivity, the results from series with condensed vegetable tannins (mimosa) and non-ionic complexes of [illegible]

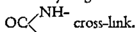
fixed by hydrogen bonding of the polyphenolic structures on the carbonyl oxygen of the keto-imide group (Gustavson, 1946a; 1947a, 1950a; 1954c, e; Lanham and Pankhurst, 1956). The very [illegible]

amides (Gustavson, 1954d, 1955c). Hence, they are probably not reacting with the keto-imide linkage of collagen. Accordingly, the increased fixation of these chromium complexes by heat-denaturation of collagen and by its pretreatment with lyotropic agents (urea, for example) cannot be due to the freeing of co-ordination sites on the —CO.NH— linkages. By O-acetylation of collagen, the irreversible binding of mimosa tannins by collagen is greatly increased, from 60 to 95–105 per cent, on the basis of protein. On the other hand, the fixation of the non-ionic chromium complexes is

zone of collagen, e.g. pH values about 5, the binding of this dye apparently occurs mainly by the OH groups of the substrate, the ampho-ionic character being less apparent. As an example, the following set of experiments is given. Portions of hide powder equal to 500 mg. of collagen were shaken for 24 hours in 20 ml. portions of 0.01 per cent solution of the dye (= 2 mg. dye). The filtrates were drawn off and the stock washed and shaken three consecutive times with 40 ml. of water. The filtrate and the washings were made up to 200 ml. The amount of unfixed dye was determined colorimetrically in the Spekker absorptiometer. The amount of dye removed by the substrate was obtained by difference. The irreversible fixation of the Benzopurpurine 4B was for ordinary hide powder 0.40 per cent on the weight of collagen, for the same hide powder after heat-denaturation (for 1 minute in 70° water) 0.48 per cent and for the exhaustively acetylated hide powder 0.17 per cent only.

The irreversibly fixed dye was stable to saturated solution of phenol upon prolonged treatment at room temperature, whereas 6–8 molar solutions of urea stripped the dye almost completely upon two consecutive extractions at 20° C. These findings indicate hydrogen bonding to be chiefly responsible for the attachment of the dye to the collagen.

These data indicate the importance of the hydroxyl groups for the non-ionic reactivity of collagen. They provide also evidence for the existence of a part of the hydroxy groups internally compensated which makes them less reactive. By denaturation these cross-links are severed and the increased reactivity of the denatured collagen to hydrogen-bonding agents may be conceived to be due to the new sites of hydrogen bonds which are set free by breaking the —OH - - -



HIGH CONCENTRATION GRADIENTS SET UP AT THE INTERFACE: COLLAGEN–SOLUTIONS OF ELECTROLYTES

In elucidating the reactions in a two-phase system, such as collagen *in aqueous solution, it is generally considered* that for systems in which the membrane potential (Donnan effects) have been eliminated, or do not exist (as in irreversible systems), the concentration of the solute in the internal solution of the solid phase is equal to the

concentration of the solute in the external solution. Results of recent investigations of gelatin gels (Jelley and Pontius, 1955), reacting with solutions of acid dyestuffs, and experimental studies on the fixation of cationic chromium complexes by collagen from solutions of basic chromium chlorides (Gustavson, 1957) show to all evidence that this assumption is not valid In fact, very large [illegible]

amino-naphthalene-diazotoluene, is enormously greater in the surface of the gelatin gel than in the dye bath The concentration in [illegible]

lyotropic effect of the concentrated solutions (Gustavson, 1926a) [illegible]

face is provided by data from the fixation of cationic complexes of basic chromium chlorides by collagen (Gustavson, 1957). A solution of the 67 per cent acid chromium chloride was equilibrated at a concentration of 1 equiv. Cr per litre. Its composition corresponded to the empirical formula. $Cr_2(OH)_2Cl_4.2NaCl$. By ionic exchange, the chromium was found to be entirely positively charged (Gustavson, 1924; 1944; 1946b). All the chlorine was found in the filtrate from the cation exchanger. Hence, *no* complexly bound chlorine was present. In the original stock solution at a concentration of 250 gm. Cr_2O_3 per litre, an amount of 5 per cent of the total amount of chromium was present as non-ionic complexes. The figures for the acidity of the complexly held Cl-groups (*chloro-groups*) varied widely in the range from 15 to 30 per cent. In view of the lability of the chloro-groups (on dilution), this large spread of the values is understandable. It is indicated that the solutions of great concentration of the chromium chloride consist mainly of complexes containing two Cl-groups in the binuclear complex (2 atoms of chromium), i.e. it is the 67 per cent acid dichloro-diol-chromium chloride $(\underset{Cl}{Cr}\overset{OH}{\underset{HO}{\diagup\diagdown}}\underset{Cl}{Cr})Cl_2$. On tenfold dilution, the chlorocomplex

is evidently rapidly and completely decomposed; the final product being the $(\mathrm{Cr}\langle^{\mathrm{OH}}_{\mathrm{HO}}\rangle\mathrm{Cr})\mathrm{Cl}_4$ compound.

The entrance of Cl-groups into the chromium complex by the augmentation of the *chrome concentration* is an item of particular importance for the experiment to be discussed. Portions of hide powder were equilibrated with the aged solution of the 67 per cent acid chromium chloride containing 25 gm. $\mathrm{Cr_2O_3}$ per litre, in which *no* chloro complexes were present. This treatment was repeated; the total duration of the tannage being 8 days, with final pH values of 2.9–3.0. The chromed hide powder had its binding capacity for these chromium complexes completely satisfied. It contained 4.10 mg. equiv. of Cr and 2.12 mg. equiv. hydrolysable Cl per gm. collagen. By equilibrating the tanned hide powder, after removal of uncombined chromium salt by washing, at pH 8.2 (4 per cent pyridine solution) and at pH 5, the distribution of the bound chloride was estimated. The Cl-groups may be present as (1) protein-bound acid (HCl); (2) complexly bound in the chromium complex (*chloro*-groups) which are stable to the treatment with 4 per cent solution of pyridine on 1–2 hours' duration; and (3) Cl-groups present in electrostatic compensation with the unipointly fixed chromium complexes ('Gegen-ion'). The original chromed hide powder containe 2.12 mequ. Cl. per gm. collagen, which was distributed as follows (mequ. per gm. collagen): 0.86 protein-bound Cl; 0.55 Cl as 'Gege ions (associated electrostatically with Cr) and 0.71 as comple bound Cl (*chloro*-groups). The composition of the fixed cat corresponded to the formula $(\mathrm{Cr_2(OH)_2Cl}){<}$, or an acidit 17 per cent.

Some intricate and puzzling inconsistencies and discrepan old standing are given a satisfactory explanation by this ne ception of the reaction. Far back in the 'twenties, Stias Balányi (1927), and the present author (1927b) found no co bound chloride in dilute solutions of the 67 per cent acid ch chlorides of the type employed (prepared by the additio mole of NaOH to one mole of the Bjerrum salt (Cr $\mathrm{Cl_2}$(C $\mathrm{2H_2O}$). The first-mentioned workers employed concentr measurements of the chloride ion and the last-mentio gator the ion exchange technique. However, by analysi powder, tanned with the same solution of the basic chr ride which was found to be free from chromium co

complexed Cl, by means of the pyridine method as well as by the [illegible]

ferred fixation of cationic complexes of the type $(Cr(OH)_2Cl)<$ by collagen, on the basis of the available experimental material and the concept of the mechanism of the reaction generally accepted. However, in view of the formation of chloro-chromium cations in solutions of basic chlorides being favoured by increasing the concentration of the compound (Gustavson, 1927b; 1944) and moreover, with due consideration of the tremendous enrichment of the concentration of solutes at the boundaries of the solid protein-aqueous solution found in the investigations by Jelley and Pontius (1955) just referred to, this apparent paradox is removed. By the increased concentration of the chromium compound in the protein, chloro-diol-chromium cations may be formed which in this form combine with collagen.

This example may appear to be rather far-fetched and not to belong to the problems under discussion in the present symposium. However, some excuse is the fact that it provides a demonstration and a proof of the setting up of very great concentration levels of the reacting solute at the interface between the collagen and the aqueous solution. It constitutes evidence for a phenomenon that is likely to enter prominently into the behaviour of collagen and connective tissue. By employing the tracer technique, this interesting phase of the reaction of collagen and solid proteins generally with electrolytes could probably be advantageously attacked.

THE POSSIBILITY OF '*IN VIVO*' TANNING OF COLLAGEN

Very little positive information is at hand concerning tanning processes in organs of higher vertebrates including man, although the importance of such hardening of protein structures of invertebrates is firmly established. For a general review, see Gustavson (1956b). Modifying of the proteins of connective tissue by self-tanning is one possibility. Thus, by splitting-off of certain reactive groups of collagen, such as strongly polar side chains, the hydration of

the structure should be lowered, and the stability of the structure should be expected to be improved. On the other hand, the elimination of the residues of the amino acids which are instrumental for the cross-linking and organization of the protein, such as the hydroxyproline residue, would be expected to have the reverse effect on collagen, i.e. impair its stability. It appears that the abnormal collagenous tissue formed in rheumatoid arthritis is low in hydroxyproline. This degenerated collagen shows a lower degree of stability and is also more soluble in alkali than ordinary collagen. In view of the importance of the hypro residue for the structural stabilization, as evident from the discussion in the foregoing sections, it would appear that *in situ* alterations of collagen affecting the particular chain in the trihelical protofibril in which the hypro-residue is laid down primarily, for instance by partial removal of such a polypeptide chain, should markedly impair the stability of the protein structure (Cowan *et al.*, 1955; Burton *et al.*, 1955; Reed *et al.*, 1956).

The properties of collagen may be modified also by cross-linking *in situ* by a secondarily formed group, possessing tanning and cross-linking potency, in the polypeptide chain without removal of the side chain. Thus, the residues of the aromatic amino acids are able to take on a quinoid character, thereby creating potential cross-linking facilities. This type of self-tanning appears to be of wide occurrence and of great importance among arthropods (Blower, 1947; Brown, 1950; Dennell and Malek, 1956). Such alterations by cross-linking and hardening of the protein structure would make it more rigid and resistant. However, it would concomitantly imply a lowered degree of elasticity which latter impairment would be disastrous for the primary function of the tendon, for instance.

The occurrence of the ordinary modes of tanning for modifying the properties of collagen in tendon and connective tissue generally is a possibility which should not be altogether ignored. In the first place, the partaking of tanning and hardening by aldehydes and unsaturated fatty acids, as well as by steroids, merits attention, a first pointed out by Rudall (1946) in discussing the hardening o epidermin and protein structures of insects. Pathological alteratio of collagen and changes on ageing of the body may well invol cross-linking of the protein by aldehydes such as methylglyox (pyruvaldehyde), an intermediate in the metabolism, and by hig conjugated glycerides or fatty acids or their peroxides. Increa

formation of such compound may occur on disturbance of the enzymatic processes. The optimum reactivity of these compounds [illegible]

undergoes on ageing of the human body has been mentioned (Gustavson, 1947b). In both instances, the changes are alike: the degree of hydration and the water-retaining capacity of the structure are reduced. Further, the degree of elasticity of the tendon is markedly impaired.

[illegible]

tendon. In tendons from individuals who had died from certain severe diseases, the function of the tendons was greatly impaired. The extension diagrams and the stress-strain curves obtained by [illegible]

of structure, such as dehydration, lessened water-retaining capacity, and deposition of organic and inorganic material. Cross-linking by methylglyoxal, for instance, has been mentioned as an additional factor (Gustavson, 1947b). Since very minute amounts of this aldehyde only will suffice for extensive stabilization of the collagen lattice and to modify the protein profoundly, it would be very difficult to prove the case by chemical analysis. However, it appears that the possible occurrence of such processes would be worthy of the attention of the medical specialist.

SUMMARY

The importance of the hydroxyproline (= hypro) residue for the stabilization of the collagen structure, particularly as to its hydrothermal stability, is discussed in the light of new experimental data

and in relation to recent models of the collagen helices. It has earlier been experimentally indicated that the hydrothermal stability of the skins of various mammals and fishes, as measured by the temperature of instantaneous shrinkage of collagen, is augmented by increased content of hypro. Thus, collagen of teleostean skin shows low content of hypro (6-10 per cent) and low values of the

The effect of exhaustive N- and O-acetylation of the collagen of bovine skin on its shrinkage temperature and on its affinity for and binding capacity of various agents of ionic as well as co-ordinate reactivity is discussed. The results are interpreted as indicating the

imide link and for the hydroxy group of collagens are considered as well as the effect of hydrothermal denaturation of collagen upon its reactivity.

The building up of highly concentrated solutions of the ionic reactants at the interface of the solid phase in the two-phase system

... data from the ... changes in ... chromium complexes on their fixation by the hide protein, as compared to the composition of these complexes in the external solution, provide evidence for the enrichment of the concentration of the solute on the surface of collagen. From the concentration profile of gelatin gel on its fixation of sulphonic acid dyestuffs, a drastic jump in the concentration of the dye at the interface of the gel is also found. *Such concentration gradients might be set up on the reaction of*

pounds by collagen (tendon) is suggested to be ...

tion.

ACKNOWLEDGMENT

Part of the researches reviewed have been supported by research grants from *Statens Tekniska Forskningsråd*, most gratefully acknowledged

Garverinaringens Forskningsinstitut,
Stockholm, Sweden

GROUP DISCUSSION

DR. BEAR drew attention to the X-ray diffraction differences between [illegible]

[illegible]

should be a slightly vulcanized protein, to use Dr Astbury's term.

DR. BEAR said that in view of the recent 3-chain models for collagen molecular structure, one should discriminate between *inter*molecular cross linkages and interchain *intra*molecular cross linkages. One of the molecular models allows intramolecular bonds involving hydroxyproline more readily than another which favours intermolecular bonds. The latter seems to be in better agreement with the diffraction data. Workers now consider that the gelatinization of molecularly dispersed collagen should be studied and attempts made to see if there is any correlation of hydroxyproline content with the thermal shrinkage temperatures of these molecules themselves as well as with thermal contraction temperatures of the tissues

DR. GROSS said that it was difficult to correlate the properties of all collagenous fibres with their structure as they are probably often not simple proteins but mixtures. For example, trypsin-purified elastoidin has a tyrosine content of 6 per cent. After autoclaving, the gelatin dissolved out contained 1 per cent tyrosine but there was an insoluble residue con-

and in relation to recent models of the collagen helices. It h[as] earlier been experimentally indicated that the hydrothermal stabilit[y] of the skins of various mammals and fishes, as measured by the temperature of instantaneous shrinkage of collagen, is augmented by increased content of hypro. Thus, collagen of teleostean skin shows low content of hypro (6-10 per cent) and low values of the shrinkage temperature (35-55° C.), whereas the collagen of mammalian skin contains larger quantitites of hypro (12-13 per cent) and markedly higher shrinkage temperature (60-70° C.).

The effect of exhaustive N- and O-acetylation of the collagen of bovine skin on its shrinkage temperature and on its affinity for and binding capacity of various agents of ionic as well as co-ordinate reactivity is discussed. The results are interpreted as indicating the presence of a comparatively strong intermolecular hydrogen bond between the hydroxy group of the hypro residue and the oxygen atom of the keto-imide group on an adjacent polypeptide chain. Data on the behaviour of agents with specific affinity for the keto-imide link and for the hydroxy group of collagens are considered as well as the effect of hydrothermal denaturation of collagen upon its reactivity.

The building up of highly concentrated solutions of the ionic reactants at the interface of the solid phase in the two-phase system of collagen-solutions of electrolytes is indicated by data from the reaction of basic chromium chlorides with collagen. The changes in the complex composition taking place in the cationic chromium complexes on their fixation by the hide protein, as compared to the composition of these complexes in the external solution, provide evidence for the enrichment of the concentration of the solute on the surface of collagen. From the concentration profile of gelatin gel on its fixation of sulphonic acid dyestuffs, a drastic jump in the concentration of the dye at the interface of the gel is also found. Such concentration gradients might be set up on the reaction of collagen with dilute solutions of electrolytes, under physiological conditions, creating conditions favourable for hydrotropic effects on the protein component.

Further, the possible occurrence of changes in collagen by ageing, or under certain pathological conditions, due to the formation and binding of aldehydes, such as pyruvaldehyde and similar compounds by collagen (tendon) is suggested to be worthy of consideration.

ACKNOWLEDGMENT

Part of the researches reviewed have been supported by research grants from *Statens Tekniska Forskningsråd*, most gratefully acknowledged.

Garverinaringens Forskningsinstitut,
Stockholm, Sweden

GROUP DISCUSSION

DR. BEAR drew attention to the X-ray diffraction differences between [illegible]

[illegible]

should be a slightly vulcanized protein, to use Dr. Astbury's term

DR BEAR said that in view of the recent 3-chain models for collagen molecular structure, one should discriminate between intermolecular [illegible]

[illegible]

the tissues

DR. GROSS said that it was difficult to correlate the properties of all collagenous fibres with their structure as they are probably often not simple proteins but mixtures. For example, trypsin-purified elastoidin has a tyrosine content of 6 per cent. After autoclaving, the gelatin dissolved out contained 1 per cent tyrosine but there was an insoluble residue con-

and in relation to recent models of the collagen helices. It has earlier been experimentally indicated that the hydrothermal stability of the skins of various mammals and fishes, as measured by the temperature of instantaneous shrinkage of collagen, is augmented by increased content of hypro. Thus, collagen of teleostean skin shows low content of hypro (6-10 per cent) and low values of the shrinkage temperature (35-55° C.), whereas the collagen of mammalian skin contains larger quantities of hypro (12-13 per cent) and markedly higher shrinkage temperature (60-70° C.).

The effect of exhaustive N- and O-acetylation of the collagen of bovine skin on its shrinkage temperature and on its affinity for and binding capacity of various agents of ionic as well as co-ordinate reactivity is discussed. The results are interpreted as indicating the presence of a compara[illegible]
between the hydroxy [illegible]
atom of the keto-imic [illegible]
Data on the behaviour of agents with specific affinity for the keto-imide link and for the hydroxy group of collagens are considered as well as the effect of hydrothermal denaturation of collagen upon its reactivity.

The building up of highly concentrated solutions of the ionic reactants at the interface of the solid phase in the two-phase system of collagen-solutions of electrolytes is indicated by data from the reaction of basic chromium chlorides with collagen. The changes in the complex composition taking place in the cationic chromium complexes on their fixation by the hide protein, as compared to the composition of these complexes in the external solution, provide [illegible]

centration of the dye at the interface of the gel is also found. Such concentration gradients might be set up on the reaction of collagen with dilute solutions of electrolytes, such as weak organic acids, under physiological conditions, creating conditions favourable for hydrotropic effects on the protein component.

Further, the possible occurrence of changes in collagen by ageing, or under certain pathological conditions, due to the formation and binding of aldehydes, such as pyruvaldehyde and similar compounds by collagen (tendon) is suggested to be worthy of consideration.

it shows a beautiful helical structure with small angle episcopic illumination.

It is curious that the periodic or helical structure of collagen fibres, so well seen under the electron microscope and also deduced from X-ray analyses, has not been recognized in the microscopic field or

(1921) described helices in the mouse tail tendons, and Heringa (1926) with his co-workers described them in skin and in tendons. He spoke of 'spiral arrangements'. His conclusions were drawn from hygroscopic torsions of bundles of tendons and from other optic phenomena Nauck (1931) working on whole human and other tendons saw 'waves' and 'periodical double lines, 13 per mm.' Heringa's measurements were similar. Lerch (1950, 1953) spoke of the macroscopic structure of tendons, which is like a cable with large helical windings The helical structure can not be seen with transparent illumination. It can easily be demonstrated if the tendon is placed in Ringer's solution on a slide and illuminated episcopally under a small angle.

With a double

beam of light falls on the fibre at an angle of 90°. Turning the slide, the helices disappear completely at 180°.

The microscopical helical structure of neighbouring tendon fibres lie in different planes. This makes it possible to recognize whether one is dealing with a single fibre or a bundle of two or more. Bundles of fibres are again helically twisted around each other like a cable (Fig. 1).

These thin fibres lose their water by evaporation extremely quickly even during their preparation. Dried fibres show no helices, but they appear immediately if the fibre is transferred to water or to a salt solution.

The analogy of this helical structure with the descriptions based on X-ray analyses (Astbury and Sisson, 1935; Bear, 1953; Springall, 1954; Hall *et al.*, 1955; Randall, 1954; Robinson and Watson, 1952; Ramachandran and Kartha, 1954) and electron microscopic pictures is striking. Of course, it must be realized that we are working here in an order of magnitude several thousand times larger. One may

THE AGEING OF COLLAGEN

F. VERZÁR

I should like to apologize for returning to macroscopic observations at a time when X-ray and electron microscopy have contributed so much to our understanding of the structure of connective tissue in general and of collagen in particular. I hope that the present work will serve at least as a model for the conclusions reached by modern physico-chemical methods.

It seemed to me important for the study of natural collagen to choose a simple source of material, and if possible one not mixed up with other tissue ingredients. Whole tissues, like skin, contain several similar substances, collagen fibres, elastic fibres, argyrophil fibres, ground substance and probably other intermediary substances. Further, since we now know that these substances can be transformed into each other (Burton *et al.*, 1956), there is the risk of transforming artificially one into the other, e.g. when preparing collagen-free elastin. Simpler methods may, therefore, sometimes have advantages. Thus we turned to the single fibre of the rat's tail tendon (Verzár, 1955). Ewald, as early as 1909, used mouse tails, Partridge (1948), Banga (1953) and others have used the rat's tail, Weir (1949), Weir and Carter (1950) and many others kangaroo-tail tendons. We have used an even more simple structure. the single tendon fibre. In the fully grown adult rat these fibres have a remarkably constant diameter and sub-macroscopic structure and are therefore especially convenient for comparative work.

These tendon fibres have to transmit the pull of the muscles proximal to the base of the tail, to the distal joints of the tail. Therefore, they should not be extensible. They are what one might call with all due respect for a complex protein structure, pure collagen fibres.

[illegible] hole in the skin, one can [illegible] out bleed- [illegible] have a diameter of 0.15 mm. and a weight of 4-5 mg [illegible] This is our material. That it is a single unit is controlled not only by the above three characteristics, but also by examination under the microscope where



Fig. 1
Helically packed tendon fibres from the rat's tail



Fig. 2
Thermic contraction of a rat's tail tendon fibre
(a) Fresh single tendon fibre at 20° C in Ringer's solution
(b)–(f) Consecutive stages of the same fibre by heating up to 62° C

This latter substance, which constitutes the transparent fibre and which we call here elastic protein, seems to be identical with metacollagen (Banga *et al.*, 1954, 1955) and with elacin of former authors

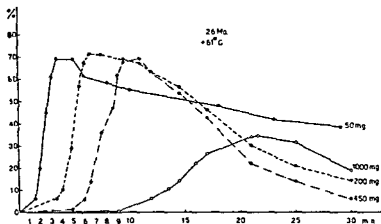
It is known that weak acetic acid dissolves mucopolysaccharides from the collagen fibre (Nageotte, 1927; Loewen, 1955a, b). Thus it is probable that the helical structure is a mucopolysaccharide, cross-linked with the elastic protein fibrils, the metacollagen. The mucopolysaccharide prevents the action of elastase on the natural collagen fibre. It has been shown by Banga that only a small

linkages which inhibit in the natural fibre the contraction of the elastic protein fibres. If heat, acid or proteinase is allowed to act for a longer time, more cross-links are broken, and the fibre becomes completely elastic. Thus it seems that our observations identify mucopolysaccharide or rather mucoprotein as the substance of the helical structure cross-linked with an elastic protein.

Certain observations on the effect of thermic contraction on these tendon fibres show that inorganic ions also play a part. If tendon fibres are bathed in distilled water for several hours or days and then heated, the thermic contraction becomes smaller and the induction time much longer. If one transfers such fibres to a 0 9 per cent NaCl solution and then heats them to 62° C, the thermic contraction immediately becomes normal in strength and time. While Na^{++} ions have thus action, Ca^{++} ions have no such influence in physiological concentrations. We also did not succeed in changing in a reversible way the thermic contraction after treatment of the tendons with a calcium ion-exchanger. Possibly calcium is more important for the binding with elastin than with collagen.

somewhat less extensible than that of young animals (...
1893; Roy, 1880; Remington, 1945; Banfield, 1952). Nor is it possible to see microscopic differences. Electron microscopic studies have shown that the interfibrillar ground substance decreased in tendons of aged animals (Schmitt *et al.*, 1942; Schwarz and Dettmer, 1954; Rollhauser, 1950; van den Hooff, 1952; Bahr, 1951; *Gross, 1952; Küntzel, 1941*).

Remarkable changes can be shown in the thermic contraction of



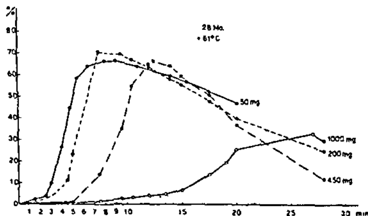


FIG. 4
Thermic contractions with different weights at 61° C in 5-, 5½-, 26-, and 28-month-old rats' tail tendon fibres.
Abscissa Minutes.
Ordinate Percentage of contraction of original length of fibre
Note the long induction times with larger weights especially in the tendons of the old animals.

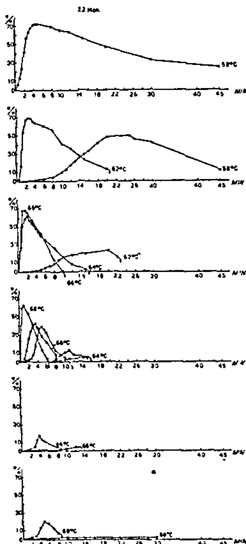


FIG. 5

Thermic contraction of 6-, 9- and 22-month-old rats' tail tendon fibres at different temperatures and weights.

Abscissa: Minutes.

Ordinate: Percentage of contraction of original length of fibre.

Note: Lower temperatures have longer induction time; larger weights need higher temperature, older animals' tendons lift larger weights at higher temperatures.

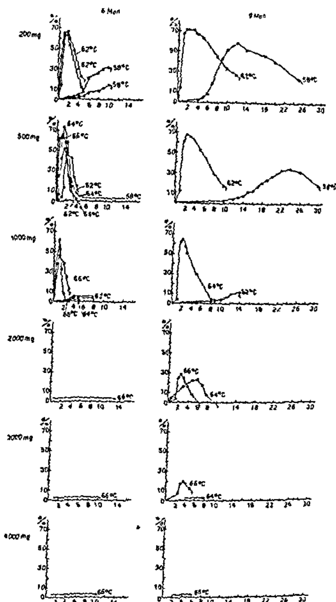


Fig. 5

Thermic contraction of 6-, 9- and 22-month-old rats' tail tendon fibres at different temperatures and weights

Abscissa. Minutes.

Ordinate: Percentage of contraction of original length of fibre.

Note: Lower temperatures have longer induction time, larger weights need higher temperature, older animals' tendons lift larger weights at higher temperatures.

ross-linkages the shortening of the contractile protein fibres. 'he destruction of the helical band is the destruction of cross-nkages and the elastic protein then contracts.

This by itself could not explain how the fibre of old animals can ... times greater weights than the tendon fibre of the young

...-linkages are present in the old fibre than in the young one, as

lcohol, he comes to the following analysis. 'In the case of aged rats a considerable part of the collagen must be present as almost completely stretched molecular chains, these being cross-linked to form a three-dimensional network. In young individuals practically the

Elevation of temperature gives the chain molecules a certain amount of freedom of Brownian motion. In this state the difference between young and old collagen shows itself. In the case of young individuals (lacking cross-linking) no or only weak contractile force of the macroscopic sample is observed, the individual chain molecules reassuming at 62° C, by Brownian motion, each one by itself, its most probable shape in the medium In the case of the old individual (cross-linking of the chains), the chain molecules transfer the change of their shape to the macroscopic sample giving rise to a contractile force of the latter At still higher temperatures, the cross-linking collapses in all cases . . . Thus the differences in the elasto-mechanical

conditions being identical. Tendons of the same animal show thermic contraction with a larger load at a higher temperature. With a small load the induction time is shorter and the height of the contraction is higher. With only 100 mg. load already at 54° C, after a very long induction time, a small contraction may occur (Fig. 4).

This is also to be applied to different ages. The tendons of old animals contract with greater loads and do so at temperatures at which the tendons of younger animals do not contract (Fig. 5). If the comparison is made with small equal loads, young animals' tendons may not show any thermic contraction while old animals show it at 54° C.

Furthermore, as has already been said above, the so-called reversibility of thermic contraction is only the result of the elasticity of the tendon fibre which appears after thermic contraction. There are again remarkable differences between this so-called reversibility in young and old animals' tendons. Small weights reverse the contraction much less and more slowly in the old than in the young fibre (Fig. 5). A weight of 200 mg. may completely and quickly reverse thermic contraction in a young animal, while the tendon of an old animal may remain for a long time contracted with such a small weight. It will immediately be elongated, however, if the contraction is made with a weight of 1000-2000 mg.

These changes in the thermic contraction are such exact signs of the age that it becomes possible to judge the age of an animal on the basis of thermic contraction of a tendon fibre which was extracted under narcosis from the animal's tail. There are differences between the fifth and the tenth month, and much greater differences between the tenth and eighteenth and finally the thirtieth month of the animal's life.

After having described these physical differences in the behaviour of single tendon fibres in animals of different age, we shall consider the physico-chemical explanation.

To quote Gustavson (1956, p. 202): 'The contraction of the collagen fibre is generally considered to result from the weakening or dislocation of the interchain cross-links of the collagen fibrils. Heat interferes with cross-links.'

If the supposition which we made from the comparison of the optical, mechanical and chemical facts is right, then the optically visible helical bands represent mucoproteins. These inhibit by

cross-linkages the shortening of the contractile protein fibres. The destruction of the helical band is the destruction of cross-linkages and the elastic protein then contracts.

This by itself could not explain how the fibre of old animals can [illegible]

that the helical structure goes through the whole diameter of the fibre, it may be that cross-linkages with mucoproteins are responsible for these interfibrillar cross-linkages. But even then it seems that after the destruction of the whole helical structure, in the transparent stage, when the fibre is completely elastic, still more cross-linkages are present in the old fibre than in the young one, as judged by the lesser elasticity of the former.

I have to thank Professor Werner Kuhn for a discussion of these problems (1956). On the basis of a comparison with his studies on artificial systems, especially on polyacrylic acid with polyvinyl [illegible]

any changes of the shape of the molecular chains are impossible due to the van der Waals forces (similarity to stretched frozen rubber). [illegible]

macroscopic sample is observed, the individual chain molecules reassuming at 62° C, by Brownian motion, each one by itself, its most probable shape in the medium. In the case of the old individual (cross-linking of the chains), the chain molecules transfer the change of [illegible]
for [illegible]
col [illegible]
[illegible]

The idea that an increase of cross-links appears with age and is

mainly responsible for ageing, was discussed by Bjork
facts of literature. While he collected only indirect p
sibility that ageing in general might mean an increase
ages between filamentous proteins was discussed in
way. King (1946) has also discussed the decrease of th
arteries in old age with application of the statistical mecha
of elasticity of high polymers. He came to the conclus
changes of elasticity are the result of the degree of cros
protein filaments which increases with age.

Wood in 1954, quoting the former work, wrote: 'It s
sible that if ageing is accompanied by cross-linking . . . a st
mechanical properties of the tissue might help to eluc
process.' Without having known about these earlier ideas,
to me that our work has produced mechanical proof for the
of cross-linkages of collagen fibres with age.

GROUP DISCUSSION

DR. BEAR said that Küntzel found that the waviness of fibrils in t
could be observed less clearly if the tissue had dried, and wondered
Verzár had studied the effect of drying

DR. VERZÁR replied that dried fibres do not show the helical struc
This appears when the fibres are immersed in water or Ringer's solu

DR. BOWES had observed similar helical banding on a larger scale w
tendons were immersed in dilute acetic acid. As the acid penetrated
the fibre began to swell opaque bands appeared but lat
the tendon became translucent

DR. G

tar
clu
Gu
with
agre ... with Dr. Verzár's explanation ... linkage ... nothing to do
refe ... to the work of Stucke who fo ... of cross linkage and
cr ... the age of 20-30 years an ... ges. He f
ir ... with the view that cross ... of tendons
n ... with the tensile streng ... se sharply.
ed if the helical stru ... collagen c
cal distortion of th ... c.
ta ... said he had checke ... Dr. Ve
SON wondered if ... remov
und it
om

caused by diffraction effects due to the optical method employed and asked if similar phenomena had been observed in polarized light. She said [illegible]

was important to remember that the tendon is a tissue (hence contains other fibrous components of various orientations) before drawing conclusions from observations such as Dr Verzár had shown. He mentioned the possibility of mechanical effects occurring during removal of the tendon from the tail.

DR. GILLMAN said that it was possible to observe striations in rat-tail tendon *in situ* before it had actually been removed from the tail. In relation to the suggestion by Dr. Verzár that elastin-like fibres are formed during contraction and relaxation, DR. HALL asked if any evidence had been obtained of material being extracted from the fibres. DR. VERZÁR said he had done no work on this.

In reply to a question from Dr. Hall, DR. VERZÁR said that treatment with Banga's mucoproteinase gave the same results as treatment with low concentrations of acetic acid.

mainly responsible for ageing, was discussed by Bjorksten in 1951 on facts of literature. While he collected only indirect proofs, the possibility that ageing in general might mean an increase of cross-linkages between filamentous proteins was discussed in an interesting way. King (1946) has also discussed the decrease of the elasticity of arteries in old age with application of the statistical mechanical theory of elasticity of high polymers. He came to the conclusion that the changes of elasticity are the result of the degree of cross-linkage of protein filaments which increases with age.

Wood in 1954, quoting the former work, wrote: '*It seems possible that if ageing is accompanied by cross-linking . . . a study of the mechanical properties of the tissue might help to elucidate the process*.' *Without having known about these earlier ideas, it seems* to me that our work has produced mechanical proof for the increase of cross-linkages of collagen fibres with age.

GROUP DISCUSSION

Dr. Bear said that Kuntzel found that the waviness of fibrils in tendon could be observed less clearly if the tissue had dried, and wondered if Dr.

al structure.

r's solution.

scale when

the tendon became translucent

found that formaldehyde

sile strength and con-

by the tanning Dr. Gustavson expressed the opinion that cross linkage had nothing to do with strength but that thermal shrinkage is a function of cross linkage and agreed with *Dr. Verzár's explanation of old age changes*. He further referred to the work of Stucke who found the strength of tendons to increase up to the age of 20–30 years and then to decrease sharply. This is

Dr. Fitton Jackson wondered if the observed

tissue preparations. Comparison of the analytical data and physical properties shows that this product is closely similar to the elastin preparation described by Stein and Miller (1938).

The elastin preparation is not soluble in boiling 40 per cent urea solution even after 24 hours' treatment, but it should be noted that [illegible]

order of 0.15–0.50 mm.

The fibres forming the dried powder from *ligamentum nuchae* were much thicker than the elastin fibres to be seen in loose connec- [illegible]

killed rat and these were fixed with the same solution. Sections were then cut and stained both by Verhoeff's and Weigert-French's procedures for elastin. The sections were finally treated with the counterstains of van Gieson and Mallory.

The fibres from the powder preparation showed the same staining properties as the elastin fibres in the areolar tissue sections; the collagen and ground substance in the latter preparations did not retain the orcein-fuchsin stain but were well demonstrated by the counterstains.

SUMMARY OF EXPERIMENTAL WORK

Since the greater part of the experimental work on which this paper is based has already been recorded (Partridge, Davis and Adair, 1955; Partridge and Davis, 1955) it seems sufficient to give the main results here in summary form.

THE COMPOSITION OF MAMMALIAN ELASTIN

S. M. Partridge, H. F. Davis and G. S. Adair

The work described in this paper has been carried out with a purified preparation of elastin powder. This substance is regarded as a chemically defined fibrous protein – it is believed to be in a state of *substantial* purity and to represent, as such, an integral component of the fibres of elastic tissue.

The yellow elastic tissue from *ligamentum nuchae* or *aorta* of cattle is known to contain, in addition to elastin, considerable amounts of

more or less intimately with the fibrous elements of pure elastin, which are *themselves largely responsible for the characteristic* physical properties of the tissue.

A procedure for the isolation of the protein 'elastin' must ensure complete removal of the other tissue components but at the same time it must not result in chemical damage to the elastin elements It has been shown (Partridge and Davis, 1950), that, with many proteins, treatment with 2 per cent acetic acid solution at 100° for several hours results in the hydrolytic cleavage of peptide bonds at specific points in the chain. Similarly the work of Courts (1954) showed that on heating a sample of gelatin at 75° for 24 hours at pH 3.0, free α-amino groups were liberated and the mean molecular weight of the sample fell from 58,000 to 7000. Boiling with dilute acetic acid as a step in the purification of elastin was avoided therefore; instead a procedure based on repeated extraction with water in an autoclave at 120° was adopted. In order to ensure the complete removal of collagen and mucopolysaccharide, *the dried powder* produced after the first course of extractions was finely ground in a hammer mill before treating for a second time in the autoclave.

grinding, consisted essentially of morphologically intact [illegible] fibres, which had the same staining properties as those to be seen in

tissue preparations. Comparison of the analytical data and physical properties shows that this product is closely similar to the elastin preparation described by Stein and Miller (1938).

The elastin preparation is not soluble in boiling 40 per cent urea solution even after 24 hours' treatment, but it should be noted that under these conditions urea solutions develop a strongly alkaline [illegible] boiling urea results in [illegible] which may be caused [illegible]

The purified preparation of elastin from *ligamentum nuchae* was a free-flowing cream-coloured powder, which under the microscope [illegible]

with a variation from 3.6 μ to 9.9 μ, whilst the lengths were of the order of 0.15–0.50 mm.

The fibres forming the dried powder from *ligamentum nuchae* were much thicker than the elastin fibres to be seen in loose connec- [illegible]

nuchae were made by embedding some of the powder in gelatin and fixing in neutral formalin solution; alongside this a number of preparations were made from abdominal areolar tissue from freshly killed rat and these were fixed with the same solution. Sections were then cut and stained both by Verhoeff's and Weigert-French's procedures for elastin. The sections were finally treated with the counterstains of van Gieson and Mallory.

The fibres from the powder preparation showed the same staining properties as the elastin fibres in the areolar tissue sections; the collagen and ground substance in the latter preparations did not retain the orcein-fuchsin stain but were well demonstrated by the counterstains.

SUMMARY OF EXPERIMENTAL WORK

Since the greater part of the experimental work on which this paper is based has already been recorded (Partridge, Davis and Adair, 1955, Partridge and Davis, 1955) it seems sufficient to give the main results here in summary form.

THE COMPOSITION OF MAMMALIAN ELASTIN

S. M. Partridge, H. F. Davis and G. S. Adair

The work described in this paper has been carried out with a purified preparation of elastin powder. This substance is regarded as a chemically defined fibrous protein – it is believed to be in a state of substantial purity and to represent, as such, an integral component of the fibres of elastic tissue.

The yellow elastic tissue from *ligamentum nuchae* or *aorta* of cattle is known to contain, in addition to elastin, considerable amounts of [illegible]

which are themselves largely responsible for the characteristic physical properties of the tissue.

A procedure for the isolation of the protein 'elastin' must ensure complete removal of the other tissue components but at the same time it must not result in chemical damage to the elastin elements. It has been shown (Partridge and Davis, 1950), that, with many proteins, treatment with 2 per cent acetic acid solution at 100° for several hours results in the hydrolytic cleavage of peptide bonds at specific points in the chain. Similarly the work of Courts (1954) showed that on heating a sample of gelatin at 75° for 24 hours at pH 3.0, free α-amino groups were liberated and the mean molecular weight of the sample fell from 58,000 to 7000. Boiling with dilute acetic acid as a step in the purification of elastin was avoided therefore; instead a procedure based on repeated extraction with water in an autoclave at 120° was adopted. In order to ensure the complete removal of collagen and mucopolysaccharide, the dried powder produced after the first course of extractions was finely ground in a hammer mill before treating for a second time in the autoclave. [illegible]

fibres, which had the same staining properties as those to be seen in

tissue preparations. Comparison of the analytical data and physical properties shows that this product is closely similar to the elastin preparation described by Stein and Miller (1938).

The elastin preparation is not soluble in boiling 40 per cent urea solution even after 24 hours' treatment, but it should be noted that

The purified preparation of elastin from *ligamentum nuchae* was a

order of 0.15-0.50 mm.

The fibres forming the dried powder from *ligamentum nuchae*

nuchae were made by embedding some of the powder in gelatin and fixing in neutral formalin solution; alongside this a number of preparations were made from abdominal areolar tissue from freshly killed rat and these were fixed with the same solution.

then cut and stained b

procedures for elastin.

collagen and ground substance in the latter preparations did not retain the orcein-fuchsin stain but were well demonstrated by the counterstains.

SUMMARY OF EXPERIMENTAL WORK

Since the greater part of the experimental work on which this paper is based has already been recorded (Partridge, Davis and Adair, 1955, Partridge and Davis, 1955) it seems sufficient to give the main results here in summary form

proteins which is studied in this paper. Six successive extractions, each for a period of one hour, were sufficient to bring dried and powdered elastin, from *ligamentum nuchae* of cattle, completely into solution. Removal of oxalic acid from the mixed extracts by dialysis through cellophane resulted in the loss of about 5 per cent of the nitrogen as small peptides.

Properties of the Soluble Protein

The non-diffusible protein was soluble in water at temperatures below 25° C to give a pale yellow mobile solution. On raising the temperature of a solution in dilute buffer at pH 4-6 a precipitate consisting of liquid droplets separated. The droplets showed no birefringence under crossed nicols and immediately dissolved again on reducing the temperature. Fig. 1 shows the temperature at which the phase separation first appeared when the pH of the solution was varied at constant ionic strength. On centrifuging at 37° C the droplets coalesced to form a lower layer of viscous liquid, and refractive index measurements showed the two liquid phases to consist of aqueous protein solutions of different concentration.

Electrophoresis Experiments

When dialysed against buffer mixtures of ionic strength 0.20 the protein showed a single symmetrical peak in the electrophoresis apparatus at all pH values in the range pH 2-9. If the ionic strength was reduced to 0.02 the protein showed a single peak in the range 4.7-7.5 but below pH 4.7 a boundary anomaly was encountered which resulted in unsymmetrical peaks, particularly in the ascending limb. Fig. 2 shows the electrophoretic mobility of the protein at ionic strength 0.02 and 0.20 when the pH was varied in the range 2-9.

Measurement of Isoelectric Point

At ionic strength 0.2 the value for the isoelectric point from electrophoretic mobility measurements was 3.9 while that from membrane potential determinations was 4.0. On reducing the ionic strength to 0.02, there was a considerable change in the position of the isoelectric point; determinations in the dilute buffer gave 4.8

from electrophoresis measurements and 4.7 from membrane potentials.

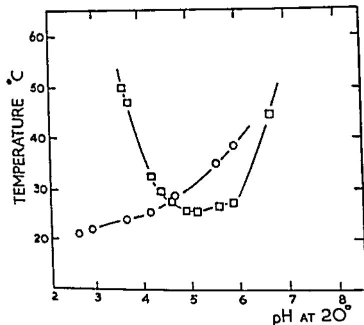


Fig 1

Temperature of first appearance of phase separation in a solution of the mixed soluble proteins from elastin. Protein concentration 0 72 per cent (w/v) □, acetate or phosphate buffer, ionic strength 0 01, ○, buffer of ionic strength 0 01 with addition of NaCl to a total ionic strength 0.10 The coacervate phase appeared on raising and disappeared on lowering the temperature

Fractionation of the Soluble Protein

Fractionation by repeated precipitation of the liquid droplets which formed on raising the temperature or by use of a highly permeable collodion membrane resulted in the separation of two components with differing physical properties. One of these, which we called α, showed the characteristic property of reversible heat precipitation. Osmotic pressure determinations for this fraction gave mean molecular weights of 60,000 to 84,000 in different preparations. The second component, which we called β, gave no precipitate at any temperature up to 100° C and had a mean molecular weight of 6000 from osmotic pressure measurements.

It was at first thought that the substances α and β may represent inhomogeneity in elastin as it exists in the tissue, and a study of the course of the hydrolysis of powdered elastin by dilute oxalic acid appeared to support the view that the substance is a two-component system. Fig. 3 shows the amounts of α and β protein contained in five successive extracts, prepared by boiling a sample of elastin powder with M/4 oxalic acid for 1 hour periods. It will be seen that the

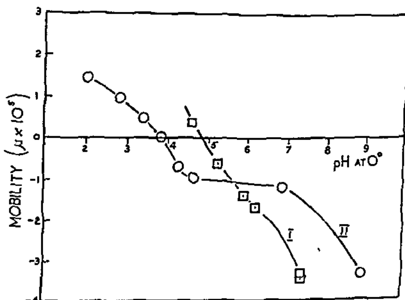


FIG 2

Electrophoretic mobility of the mixed soluble proteins from elastin. □, buffer of ionic strength 0 02, ○, buffer of ionic strength 0 02 with the addition of NaCl to total ionic strength 0 20

early extracts are very rich in β-protein (M.W. 6000) while the later extracts are rich in α-protein (M.W. 60,000–85,000). This is the reverse of what would be expected if the protein β was a product of further hydrolysis of a soluble protein of higher molecular weight, and suggests that β arises from a more easily hydrolysed component in the original tissue.

Amino Acid Composition

The question remains as to whether, if there are two components in elastin, they are of different chemical composition or whether, on

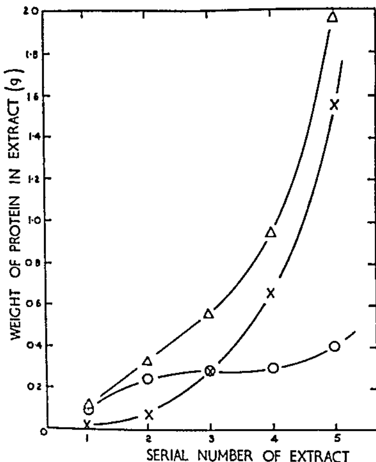


FIG 3
Extraction of 5 gm of elastin powder by heating with successive 50 ml. quantities of 0.25 M oxalic acid at 100° for 1 hour, △, gm. total protein dissolved in each extract, X, gm α-protein released, O, gm β-protein released

the other hand, they consist of basically the same kind of chain, but differ in their state of physical aggregation or degree of crystallinity. Accordingly, the amino-acid composition of the original elastin powder

TABLE I

AMINO-ACID ANALYSES OF ELASTIN AND THE PROTEINS DERIVED FROM IT BY MILD ACID HYDROLYSIS

	gm amino acid/100 gm protein dry wt.				
	Elastin from ligamentum nuchae *prep* 1	*prep.* 2	*α-Protein*	*β-Protein*	*Elastin from Aorta*
Glycine	26.7	27.0	23.2	29.0	26.2
Alanine	21.3	23.1	24.5	20.8	21.6
Leucine	9.0	8.9	8.3	8.1	9.5
Isoleucine	3.8	3.7	3.0	3.8	4.3
Valine	17.7	17.0	15.5	20.6	18.0
Serine	0.85	1.0	0.89	0.85	1.5
Threonine	1.1	1.2	1.1	0.98	1.7
Proline	13.5	12.8	12.3	13.8	14.5
Phenylalanine	6.2	6.2	—	4.6	6.2
Tyrosine	1.5	1.2	1.6	1.1	2.6
Methionine	Trace	Trace	Trace	Trace	0.53
Histidine	—	0.09	0.03	0.05	0.31
Arginine	1.3	1.2	1.2	0.93	1.9
Lysine	0.50	0.54	0.56	0.48	1.2
Aspartic acid	1.1	1.1	0.66	1.0	2.1
Glutamic acid	2.4	2.6	2.6	2.1	3.9
Hydroxyproline	1.6	1.6	1.5	—	1.7
Cystine	0.35	—	0.40	—	—

The analyses show that the amino-acid composition of the three proteins is substantially the same, and thus it appears that elastin powders, as usually prepared, may be regarded, as they have been in the past, as essentially homogeneous for the purpose of chemical investigations. Table I also includes the amino-acid analysis of

from either type of tissue are very similar chemically. Comparable data have been given by Neuman (1949) for elastin from *ligamentum nuchae* and by Lansing *et al.* (1951) for elastin from old and young aorta. It both cases the analyses were based on microbiological assay and the data indicates greater differences in amino-acid composition than were observed in this work.

Titration Experiments

... of the soluble proteins, previous

peptide

hydro-

lytic procedure releases aspartic acid and probably glutamic acid in a free state, and at the same time cleaves the protein chains at other [illegible]

show the presence of many α-carboxyl and α-amino groups in both the soluble proteins. From the titration data it is concluded that during the treatment of the native elastin to give the α- and β-proteins, about 26 and about 31 moles of α-amino residues respectively are released in 10^4 gm. of protein, with the simultaneous release of corresponding numbers of α-carboxyl residues.

Estimation of Terminal Groups

Application of the fluorodinitrobenzene technique of Sanger gave the results shown in Table II.

TABLE II

NUMBER OF MOLES OF N-TERMINAL RESIDUES IN 100,000 GM PROTEIN

	Elastin	*α-Protein*	*β-Protein*
Leucine / Isoleucine	Trace	2·6	2·9
Valine	Trace	4·0	6·6
Alanine	0·08	8·6	11·0
Glycine	0·12	8·8	12·0
Serine	0·05	Nil	Nil
Aspartic acid	0·04	0·4	Trace
Total	0·29	24·4	32·5

The small number of N-terminal residues in elastin are probably not significant since they may be due to traces of collagen breakdown products remaining in the elastin preparation after removal of collagen by autoclaving.

It is significant that the α- and the β-protein contain almost equal [illegible]

and that the larger protein molecule must be built up by the binding together of a number of such unit polypeptide chains.

fibre. The liberated collagen fibrils can easily be identified by examination with the electron microscope, since they show the characteristic cross-banded structure with major periodicity at 640 Å. According to Dempsey and Lansing the collagenous materials appear to be embedded in the elastic substance and may thus be protected from the solubilizing effects of the reagents designed to destroy them.

[illegible]

links occurs during the extraction with 0.25 M oxalic acid. The hydrolytic procedure releases aspartic and possibly glutamic acid in a free state, and at the same time cleaves the protein chains at other positions where labile peptide linkages are to be found (Adair *et al.*, 1951). Titration curves carried out on elastin and the α- and β-proteins show the presence of many α-carboxyl and α-amino groups in both the soluble proteins. From the curves the α- and β-proteins contain 26 α-amino equivalents and 30 α-amino equivalents per 100,000 gm. of protein respectively. This result accords rather well with the information obtained by application of the fluorodinitrobenzene technique of Sanger (1945) which is reported in Table II. The number of N-terminal amino-acid residues found per 100,000 gm. of protein for elastin, α-protein and β-protein was 0.29, 24.4 and 32.5 respectively. The relationship between chain length and molecular weight is brought out in Table III. The data in this table shows

TABLE III

MOLES OF N-TERMINAL RESIDUES IN AMOUNTS OF ELASTIN DERIVATIVES CORRESPONDING TO THE MEAN MOLECULAR WEIGHT DETERMINED OSMOTICALLY

	α-Protein (67,000 gm.)	β-Protein (5 500 gm.)
From titration curve	17·4	1·73
From FDNB determination	16·2	1·84

that the β [illegible]

DISCUSSION

The discovery that the product of mild acid hydrolysis of elastin contained two proteins with markedly different physical properties led to the postulation of three alternative views of the macromolecular structure of the protein comprising the elementary elastin fibre.

(1) The protein is composed of two components of differing chemical composition.

(2) The protein is composed of two components which consist basically of the same kind of polypeptide chain, but differ as a result of differences in structural configuration.

(3) The protein is homogeneous, and the production of two degradation products of different molecular weight is due to the loosening of a network by removal of parts of it as low-molecular material.

A clear decision on alternative (1) has been obtained by determining the amino-acid composition of the original elastin powder, the α-protein and the β-protein. The results given in Table I show that the amino-acid composition of the three proteins is substantially the same, and thus it appears that elastin powder as prepared in this work may be regarded as essentially homogeneous for the purpose of purely chemical investigations.

The amino-acid composition of aorta elastin, though very similar to that from *ligamentum nuchae*, shows differences in respect of certain amino acids which are outside the range of probable experimental error. These differences may arise through residual contamination of the 'purified' preparations by collagen or degradation products of collagen. Removal of the last traces of collagen from preparations of elastin powder appears to be a slow and difficult process, and inspection of the data in Table I shows that the differences in the nalytical figures for the aorta elastin could largely be explained by ssuming a certain degree of contamination by a protein of collagen-s origin. The difficulty of purifying elastin fibres becomes under-ndable in the light of observations reported by Dempsey and nsing (1954) who state that when preparations of elastin purified h hot NaOH solutions are treated with elastase, collagen fibrils liberated apparently from the internal structure of the elastin

ibre. The liberated collagen fibrils can easily be identified by examination with the electron microscope, since they show the characteristic cross-banded structure with major periodicity at 640 Å. According to Dempsey and Lansing the collagenous materials ppear to be embedded in the elastic substance and may thus be rotected from the solubilizing effects of the reagents designed to lestroy them.

As regards the molecular structure of the two soluble degradation roducts derived from the elastin of *ligamentum nuchae*, previous work has shown that a considerable degree of hydrolysis of peptide inks occurs during the extraction with 0.25 M oxalic acid. The hydrolytic procedure releases aspartic and possibly glutamic acid in a free state, and at the same time cleaves the protein chains at other positions where labile peptide linkages are to be found (Adair *et al.*, 1951). Titration curves carried out on elastin and the α- and β-proteins show the presence of many α-carboxyl and α-amino groups in both the soluble proteins From the curves the α- and β-proteins contain 26 α-amino equivalents and 30 α-amino equivalents per 100,000 gm. of protein respectively. This result accords rather well with the information obtained by application of the fluorodinitrobenzene technique of Sanger (1945) which is reported in Table II. The number of N-terminal amino-acid residues found per 100,000 gm. of protein for elastin, α-protein and β-protein was 0 29, 24.4 and 32.5 respectively. The relationship between chain length and molecular weight is brought out in Table III The data in this table shows

TABLE III

MOLES OF N-TERMINAL RESIDUES IN AMOUNTS OF ELASTIN DERIVATIVES CORRESPONDING TO THE MEAN MOLECULAR WEIGHT DETERMINED OSMOTICALLY

	α-Protein (67,000 gm)	β-Protein (5,500 gm)
From titration curve	17 4	1 72
From FDNB determination	16 2	1 84

chains containing an average of 35 residues each, the N-terminal residues being glycine, alanine, valine and leucine with a small proportion of aspartic-acid terminals.

As yet there is little indication as to the nature of the cross-links which hold the chains together. Since the α-protein *is derived from* native elastin by a process involving rupture of the peptide chains, it seems probable [illegible]

may then represent a 'resistant core' derived from the more highly cross-linked sections of the elastin fibre.

The rubber-like elasticity of the hydrated elastin fibre suggests that it is a rather disordered structure composed of bundles of randomly contorted peptide chains lying generally parallel to the fibre axis. For the greater part of their length the peptide chains must be free to take up independent thermal motion; but to account for the insolubility and the swelling properties of the fibre, the presence of cross-links at rather wide intervals must be assumed.

It is possible that such cross-links could be established by the presence of crystalline regions containing polar groups in close apposition, but there appears to be little evidence for such a structure from published X-ray diffraction studies. However, since elastin, unlike collagen, is resistant to the action of prolonged autoclaving, and shows no tendency to dissolve either in hydrogen bond-breaking solvents such as urea or in organic solvents such as phenol or cresol, it seems more probable that the primary covalency is involved in the relatively small number of cross-links present.

The only type of covalent cross-link that has so far been proved to exist in proteins is the -S-S- bridge due to two cystine half-residues. In elastin, however, cystine represents not more than 0.4 per cent of the protein dry weight, and this content would not provide sufficient cross-links to account for the stability of the structure. In order to ascertain if cystine bridges are an important factor in stabilizing the protein, elastin powder was treated with performic acid under conditions which ensured the conversion of the whole of the cystine half-residues to cysteic acid, thus breaking all -S-S- bridges. The oxidized protein remained insoluble after prolonged autoclaving, and was also insoluble in neutral 40 per cent aqueous urea and 90 per cent phenol both at room temperature and 100°. It thus appears that -S-S- bridges have little effect on the

solubility properties of the protein, and other stable cross-links must be present.

Cross-linking at the ε-amino groups of lysine or the phenolic hydroxyl groups of tryosine appears to be eliminated since it is shown that these groups are available for reaction with fluorodinitrobenzene. The amino-acid analysis of elastin and the α- and β-proteins shows them to have a remarkably low content of polar side chains. Aspartic and glutamic acid together account for 3.5 per cent of the dry protein; serine and threonine account for 2 per cent and hydroxyproline 1.6 per cent. It is possible that ester links formed between the hydroxyamino acids and such of the dicarboxylic amino-acid residues as are not combined with ammonia may account for the stability of the structure, but no evidence for the presence of such bonds has as yet been obtained. However, it should [illegible]

A further possibility remains that the peptide fabric itself is involved either in covalent cross-linking or in aggregation at the molecular level by the intertwining of helical chains. Many workers now agree that the α-helix is the basic chain configuration in α-polypeptides and the α-forms of fibrous proteins, and various model systems involving molecular aggregation with the production of 'super-helices' have been discussed by Crick (1952), Pauling and Corey (1953), Ramachandran (1956) and others. In the case of elastin, a highly ordered structure appears to be unlikely, but the production [illegible]
could be en[illegible]
conceivably [illegible]
[illegible]
h[illegible]
w[illegible]
di[illegible]
sional protofibrils. However, the possibility of inhomogeneity at a higher level of organization should not be forgotten, and the present results are not incompatible with a model consisting of two structural components at the fibril level (alternative 2).

[illegible]

Banga. They concluded that the fibres (diameter about 5 μ) were built up from many minute threads of fairly uniform thickness. [illegible]

The whole structure seemed to be cemented together and coated with a matrix material which had physical and chemical properties nearly identical with those of the fibrillar substance (cf. Bahr, 1951). The kinetics of the process of dissolution by elastase was followed by observing the liberation of Nile blue sulphate from stained elastic fibres, and the results suggested that the matrix material passed into solution at a much greater rate than the fibrillar substance. This result conforms closely with our own observations on the rate of liberation of the α- and β-proteins by mild acid hydrolysis and suggests that the fibrillar substance may be identified as the source of the highly cross-linked or branched α-protein and the matrix material as source of the β-protein which contains not more than two peptide chains linked together.

Recently Bowen (1953) has published a study of a soluble protein derived from elastin by prolonged boiling with 40 per cent (w/v) [illegible] reversible phase separation on [illegible]

out with the protein derived from urea treatment suggested that the material may exist in solution at pH 4 as a dynamic equilibrium between more than one molecular species, dilution favouring breakdown into small molecules. By extrapolation to zero concentration a value for the mean molecular weight at infinite dilution of 6870 was obtained, and Bowen suggests that this may mean identity with the β-protein produced by oxalic acid treatment (M.W., 6000). A reliable value for the maximum molecular weight approached at high concentration could not be given, but in Bowen's view a value of 84,000, corresponding with the α-protein, would be in reasonable accord with the data.

In our work with the α- and β-proteins we have not observed any tendency towards inter-conversion of the two proteins due to alterations in the protein concentration; this is well brought out in the curves relating the effect of protein concentration on osmotic pressure (Partridge, Davis and Adair, 1955). Strong solutions of the

β-protein can be stored for prolonged periods without production of

ammonia is produced and the solution becomes alkaline. Solubility in urea may be brought about by partial alkaline hydrolysis, and the *alkaline conditions may cause the rupture of interchain linkages* which are more stable under acid conditions. The difference in properties of the two types of soluble degradation product may thus be due mainly to the absence in the urea product of stable cross-bonds. Work is continuing in an attempt to obtain further information on this aspect of the problem.

Action of Elastase on Elastic Tissue and Purified Elastin

The state of combination of the polysaccharide in yellow connective tissue and its possible effects in stabilizing the protein structure has been discussed by several authors, but some confusion has arisen largely as a result of inadequate definition of the preparations of

result they suggested that pancreatic elastase is not a proteolytic enzyme, but rather a mucase Banga and Baló (1953) and Banga (1953) discussed the liberation of reducing substances during the action of elastase and about the same time Hall (1953) showed that the elastase preparations commonly used are probably complex and that at least one component has mucolytic activity. Wood (1953)

The polysaccharide can be removed in large part by extraction with alkaline 10 per cent (w/v) calcium chloride and appears to be

this and other evidence it was concluded that there is doubt as t whether the polysaccharide associated with elastin plays an import ant part in structural stability.

The results of the present work show that the elementary fibre comprising the bulk of the native ligament consist of a protein whic contains not more than 0.3 per cent of carbohydrate and insignifican amounts of ester-bound sulphate. The protein may be regarded a chemically homogeneous but it is not excluded that individual pep tide chains, comprising the protein, may constitute a 'family' o molecules in which replacement of certain amino acids, one b another, is permissible. Estimation of free α-amino groups liberate during the action of elastase on this protein (Partridge and Davis 1955) shows that enzymic degradation involves the rupture of many peptide bonds, and thus at least one of the components of 'elastase' must be regarded as a proteolytic enzyme.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of *Scientific and Industrial Research.*

GROUP DISCUSSION

[illegible] of the
Neu-
rotein,
the 6000 molecular weight particles of this fraction must be dissimilar. Dr Partridge agreed and said that this might arise from random hydrolysis.

Dr. Neuberger found it hard to see how the polypeptide chains in the alpha fraction could be joined together covalently and suggested that some reversible association, such as occurs in insulin, might occur in the alpha fraction

[illegible] of the variation of osmotic
dealing with an
hydrogen-bond

In reply to Dr. Meyer, Dr. Partridge said that 5–6 per cent [illegible]

[illegible]
in elastin.

Dr. Jackson had observed a shift of the iso-electric point of gelatin from both citrate-soluble collagen and insoluble collagen, as the salt concentration of the solution was increased. In both cases the iso-ionic point was pH 9 3 and the iso-electric point at an ionic strength of 0.1 was pH 5 1

Dr. Partridge said that it must be due to firm binding of the chloride ion by these proteins.

[illegible]

substance, and he suggested that some interchain linkage must be present in collagen

In reply to Dr. Baló, Dr. Partridge said that his elastin preparations did not contain fat but pointed out that any fats on the outside of the native fibres would have been removed during his purification procedure

In reply to Dr. Consden, Dr. Partridge said that his elastin preparation contained only 0 3 per cent carbohydrate. Though this might be involved

[illegible]

or lipo-polysaccharide associated with the outer coating might have been removed during purification.

Dr. Partridge said that the appearance of the fibres suggested that their essential structure had not been damaged by the purification procedures used.

CHEMICAL AND ENZYMATIC STUDIES ON ELASTIN

D. A. Hall[1]

Chemical studies on the structure of elastin have developed far more slowly than corresponding studies on the other fibrous component of connective tissue – collagen.

Ten years ago although characterization of elastin by histological means was commonplace (Unna, 1896), few facts were known regarding its chemical structure and these were confined to an incomplete amino-acid analysis, for example Stein and Miller, 1938. Since then electron microscopical (Wolpers, 1944; Gross, 1949 and Hall, Reed and Tunbridge, 1955) chemical (Bowes and Kenten, 1949, and Partridge and Davis, 1955) and enzymatic (Banga and Schuler, 1953; Hall, 1955 and Lansing, Rosenthal, Alex and Dempsey, 1955) examination of elastin has advanced rapidly. On the other hand the fundamental background for staining reactions has been studied mainly only in so far as the metachromatic reaction (Baló, Banga and Schuler, 1954) of the fibres is concerned and with

The studies of the past decade have, moreover, been confused by numerous apparent contradictions between the reported results of various groups of workers. It was suggested in 1951 (Hall) that adequate characterization of elastin for any analytical procedure would entail the assessment of at least two properties with a view to proving the identity and purity of the preparation. Many of the discrepancies may well be attributed to the fact that whereas one group of workers may base the identity of their preparations on chemical analyses, others consider elastin to be pure if it consists of an histologically homogeneous species.

Having electron-microscopical, histological and chemical methods available, the Leeds group have aimed at a correlation of all three

[1] Nuffield Gerontological Research Fellow, Department of Medicine, University of Leeds, England.

methods and have utilized them wherever possible for characterization of the starting material for analytical procedures.

One of the most controversial questions of elastin chemistry has been the evaluation of the role played by polysaccharide in the stabilization of the elastin molecule. Completely contradictory

insolubility on elastin or the factor conferring resistance to enzymatic attack or even the factor conferring physical stability to the

inertness and physical strength are completely unrelated. It is, therefore, possible that the removal of a portion of the naturally

tensile properties of the structure. Without unlimited facilities it is virtually impossible to examine all these facets of the problem simultaneously and therefore caution has to be exercised in specifying the particular reaction under consideration when attempting to construct a model which will explain the stability of the structure

Leeds have based their subsequent studies was first proposed in a

the case of solid enzyme. Where these are only available in solution, e.g. in more purified fractions, 1 ml. of solution containing between 60 and 70 µg. protein was added to 9 ml. of buffer. The mixture was then incubated at 37° for periods varying from 2 to 17 hours depending on the purity of the elastase preparation, and the protein dissolved estimated in the supernatant by means of the biuret reagent after the enzyme action had been stopped by immersion of the reaction mixture in boiling water for three minutes.

As can be seen, there is a marked increase in activity of the enzyme as one passes through the series of purification procedures. Electron microscope examination of the preparations obtained by treatment with acetic acid showed that all structurally discernible collagen had been removed. Chemical analyses (Hall, 1955) on the other hand, especially of preparations obtained from aorta, showed that there still [illegible]

Bowes and Kenten (1949) and more recently by Partridge *et al.* (1955) During treatment with alkali there was no marked variation [illegible]

hours' treatment. There were, however, marked changes in the polysaccharide content of the residue after varying periods of boiling with alkali. During the first two hours' boiling the polysaccharide content fell rapidly although it never reached a value below 0.2 per cent, maintaining this figure until complete dissolution ensued.

It would normally be expected that even prolonged autoclaving with distilled water (Partridge *et al.*, 1955) would be a less drastic method of collagen removal than shorter periods of autoclaving with acetic acid. That this is not so, however, can be deduced from a comparison of the polysaccharide content of elastin preparations obtained by the method of Adair *et al.* and by the present author (Hall, 1955). The only explanation would appear to be that the acidic polysaccharides concerned are less soluble at pH 2 to 3 than at pH 6, and hence the fraction containing polysaccharide remains with the residue on extraction with acetic acid.

THE ENZYMATIC SUSCEPTIBILITY OF ELASTIN PREPARATIONS AFTER DIFFERENT PERIODS OF ALKALI TREATMENT

Partially purified elastase preparations show a striking degree of dissimilarity in their reactivity with elastin preparations that have received varying degrees of alkali pre-treatment (Hall, 1955) depending apparently on the precise nature of the preparative process employed. Fig. 1 shows the type of curve obtained, relating amount of protein taken into solution to the degree of pre-treatment of the substrate at a variety of enzyme concentrations, for an enzyme preparation obtained by the precipitation at 45 per cent ammonium sulphate concentration of a pH 4.5 sodium acetate buffer extract of defatted hog pancreas. Peak activity in terms of protein passing into solution occurs after alkali treatment amounting to between 30 and 60 minutes Thereafter apparently the substrate of the enzyme concerned is either removed or altered in some way so that it is no longer susceptible to attack.

Subsequent experiments on the fractionation of such elastase preparations at various ammonium sulphate concentrations showed that the enzyme associated with this particular peak at intermediate periods of alkali treatment could be concentrated in a fraction precipitated at 20 per cent ammonium sulphate concentration (Hall, 1956).

A variety of other procedures which have been used for the fractionation of elastase have all afforded further evidence for the dual nature of the enzyme but have not been adequate for complete separation of the various components. Thus, precipitation with acetone, or adsorption and elution from alumina or from a column of powdered elastin, have provided enzyme fractions which were rich in one or other of the components, but which were not composed of [illegible]

[illegible]

thin perspex sheets forming the upper and lower surfaces of a pair [illegible]

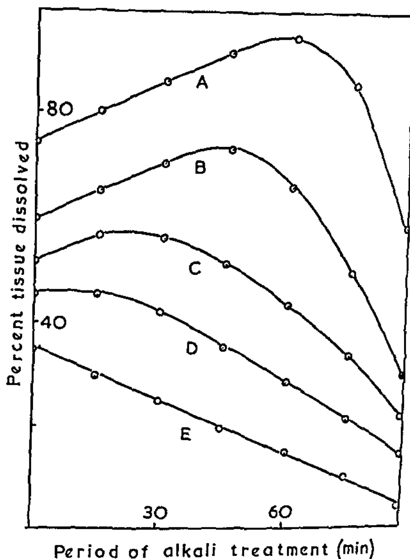


FIG 1

The relationship between the percentage of tissue dissolved and the period of alkali pre-treatment at a series of differing enzyme concentrations A, B, C, D and E refer respectively to enzyme concentrations of 100, 80, 60, 40 and 20 µg per ml of 0.1 M glycine buffer pH 8.7. 50 mg of each substrate were incubated in 10 ml. of this solution at 37° C for 17 hours.

Dyeline Reproduction of the Central Part of an Electrophoretogram of Elastase

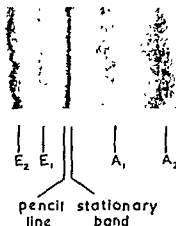


FIG. 2

The reproduction of the central five bands of an electrophoretogram of crude elastase preparation carried out in *veronal buffer pH 8.6 μ = 0.1 for 1½ hours under a P.D. of 20 volts* per cm

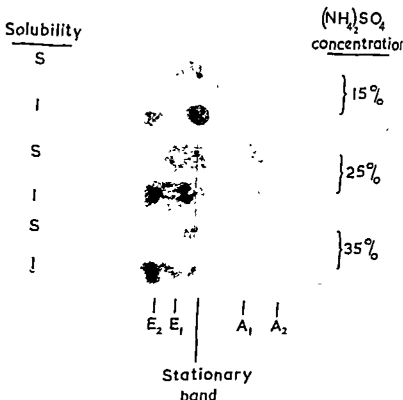


Fig 3

An electrophoretogram carried out under similar conditions to those described in Fig 2 showing the difference in composition of the soluble and insoluble (S and I) components of *three fractions obtained by an ammonium sulphate precipitation between 10 and 15 per cent,* 20 and 25 per cent and 30 and 35 per cent ammonium sulphate concentration

than that normally used. Adequate separation of serum proteins, for instance, is accomplished in $\mu = 0.1$ pH 8 6 veronal buffer in $1\frac{1}{2}$ to 2 hours, using an overall P.D. of 1000 V to give a maximum current of 40 mA.

Crude elastase preparations examined on this equipment show the presence of 10 or more bands completely separated from one another (Fig. 2). The bands are stained with Solvay Purple by the [illegible]

by heat denaturation they are lost during the application of the dye. These bands assume a grey-blue appearance as opposed to the pure blue tint of those which are sufficiently insoluble to be stained by drying at 100°. Of the 10 bands observed in crude elastase preparations, only one or two are completely removed by the various fractionation procedures. Ammonium sulphate precipitation, for instance, appears to affect the concentrations of various components so that between the two extremes of a series of ammonium sulphate precipitates, there is a gradual change in the intensity of the various bands on the electrophoretogram (Fig. 3). Only two of the bands appear to have any connection with the enzyme reaction. These are the first and second basic bands Of these the least basic E_1 when tested alone appears inactive since it does not have the power to cause protein to pass into solution, which is the normal method of assessing activity. The second band E_2, however, does have this faculty. When they are both tested simultaneously, a 50 per cent increase in the measured activity of E_2 can be observed. The optimum ratio of E_1 to E_2 is 1 to 5, further additions of E_1 having no effect on the enhancement of activity of E_2. A number of experiments have been carried out utilizing a composite eluate containing both E_1 and E_2 in the proportions in which they were originally present in the impure enzyme and it has been shown that electrophoretic separation of these two factors from the rest of the elastase preparation effects the removal of at least one proteolytic factor. This can be demonstrated in the following manner:

Relatively impure elastase preparations bring about the initial appearance of protein in solution and this increases until the whole of the elastin preparation has been dissolved. On prolonged incubation, however, estimation of protein in solution shows a decrease indicat-

ing the presence and simultaneous action of a proteolytic enzyme degrading the proteins into small peptides and amino acids which are indeterminable by the biuret technique.

E (the composite solution of E_1 and E_2) is completely devoid of this enzyme since the concentration of protein in the elastolysate remains constant for periods up to 90 hours' incubation (Table II).

TABLE II

REMOVAL OF PROTEOLYTIC ENZYMES FROM ELASTASE BY ELECTROPHORESIS

Time of Incubation hour	*Biuret Readings Crude Elastase preparation*	'E'
0 5	0 5	3 8
1 0	2	10 4
2 0	8	23 8
4 5	16	29 8
12 0	27 5	29 6
16 0	29 2	29 6
24 0	28 1	29 7
40 0	25 0	28 9
90 0	22 0	29 1

Although elastase causes protein to pass into solution and there is no evidence to preclude completely the fission of peptide linkages, it would appear that solution is accomplished without recourse to generalized proteolytic action.

... preparations
later by
activity

curve was such as to indicate the presence of two distinct enzymes. In the papers of the Hungarian workers this was exemplified by the appearance of a curve with an optimum peak associated with a shoulder at a lower pH value. It could be shown (Hall, 1955) that under certain circumstances, particularly when the substrate had received intermediate periods of alkali pre-treatment the shoulder in the regions of a pH of 7.8 would transform into a peak, so that partially purified enzyme preparations tested against alkali treated substrates gave a double-humped pH activity curve with one peak at 7.8 and the other in the region of 8.7. On purification it was found that E_1 had a single peak but that due to changes brought about by the removal of extraneous material the peak had moved to

a lower pH value in the region of 8.4 to 8.5. When examined alone, E_1, as previously stated, showed no activity in terms of dissolved protein, but the addition of small quantities of E_1 to E_2 brought about the reappearance of the shoulder at the lower pH value (Fig. 4).

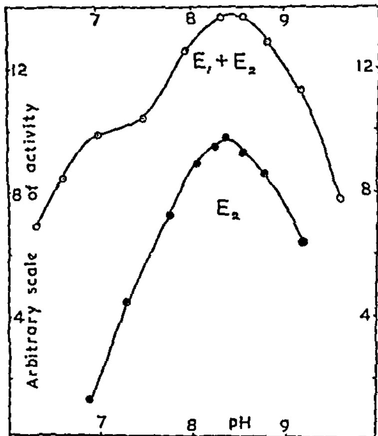


FIG. 4

Curves relating the activity of fraction E_2 and fractions E_1 plus E_2 to pH.

This could also be shown by pre-treatment of the substrate with E_1 at a range of pH values, followed by treatment with E_2. Under these conditions E_2 was found to be more active against those preparations which had had E_1 treatment in the regions of pH 7.8. The

addition to E_1 of small concentrations of E_2 permitted the solubilization to proceed. It was found that under these conditions the ratio of polysaccharide to protein liberated by E_1 containing E_2 was between 2 and 3 times as great as that liberated by E_2 either alone or in the presence of a small concentration of E_1 (Hall, 1956). This appeared to afford evidence for the involvement of E_1 in a reaction with a polysaccharide fraction of the substrate, although the fact that no polysaccharide appeared in solution without the intervention of E_2 would imply that E_2 does not sever sufficient bonds to liberate carbohydrate. The evidence, therefore, indicates that the two active fractions of elastase are respectively specific for a protein and a polysaccharide portion of the total fibre, but in both cases the enzyme is highly specific in its action, since no generalized proteolytic or mucolytic activity can be discerned.

The necessity for both protein and polysaccharide to be attacked before elastin can pass in solution affords evidence for the existence of a polysaccharide-protein complex in the fibre.

CHEMICAL EVIDENCE FOR A MUCO-PROTEIN COMPLEX

Further evidence in favour of this can be obtained by an examination of extracted fractions obtainable from whole *ligamentum nuchae* (Lloyd, 1956). After the removal of a large proportion of the mucopolysaccharide from minced ligament by extraction with hot 5 per cent sodium chloride solution a further fraction can be extracted by neutral 10 per cent calcium chloride solution. This material can be subsequently divided into two parts by dialysis. One of these is insoluble in water. This material must presumably undergo a certain amount of denaturation during dialysis since it no longer dissolves in calcium chloride solution nor will it dissolve in buffer solutions up to pH 9. However, on incubation with elastase it passes rapidly into solution and dissolution is accompanied by the release of very few free reducing groups indicating that the enzyme does not cause generalized breakdown of glycosidic linkages during reaction. Whereas whole *ligamentum nuchae* has a polysaccharide content of between 1 and 2 per cent and a preparation of elastin which has been decollagenated by treatment with acetic acid contains between 0.3 and 0.5 per cent polysaccharide, this fraction has a polysaccharide content of between 6 and 8 per cent. The amino-acid composition of the protein moiety of this preparation differs

from that of both collagen and elastin. It has a high proline content, relatively high acidic and basic amino-acid content and a hydroxyproline content intermediate between that of elastin and collagen. It could not, however, be a mixture of collagen and elastin since there are one or two spots in the region of methionine and tryptophan which could not be identified with components of either of the possible parent substances. Further analysis of the whole preparation showed that it contained about 38 per cent of reducing material on hydrolysis and about 2.4 per cent of hexosamine. Corresponding hydrolyses of a chondroitin sulphuric acid preparation give a ratio of reducing sugar to hexosamine of 13.4 to 15.2 indicating that the polysaccharide portion of the material extracted from elastic tissue was relatively devoid of hexosamines.

CONCLUSIONS

In 1952, owing to a belief that the elastase preparations then available were far purer than they have since proved to be, it was suggested that elastase attacked an outer matrix or sheath of the elastin fibre which protected an inner fibrous structure (Hall, Reed and Tunbridge, 1952). On the removal of this protective coating, the inner portion, which was assumed to be completely protein in nature, passed spontaneously into solution in the buffer.

More recent observations have done little to detract from the original concept of the elastin fibre as a dual structure and in this the chemical evidence has been amply borne out by electron microscope [illegible]

Reed, Saxl, Tunbridge and Wood, 1955). The only variation from the original concept concerns the stability of the inner fibrous protein structure. This would appear to be the substrate for the enzyme E_1, whereas the polysaccharide portion of the amorphous outer coating is the substrate for E_1.

In the earlier paper by Hall, Reed and Tunbridge (1952) it was suggested, so as to conform with the observations of Adair, Davis and Partridge (1951) and Bowen (1953) that the outer amorphous mucoprotein portion might be considered as consisting of protein units of the order of 5000–10,000 molecular weight bound together

by the small amount of mucopolysaccharide present, while the inner fibrous component was totally composed of protein and consisted of a linear polymer of this protein fraction. This theory requires very little alteration in view of the mass of evidence which has accumulated since it was first proposed, but it is possible to see how other workers using different techniques for purification of elastin preparations have removed the whole of the polysaccharide and with it, therefore, at least part of the outer coating of the fibres, revealing the inner fibrous structures on which they have subsequently performed their chemical analyses. No doubt the analyses ascribed to this material can justly be claimed to be those of elastin; but only if elastin is defined as 'a protein derived from elastic tissue by certain given purification procedures'. Such a preparation would appear to bear little relationship to elastin as defined by histologists, which calls for the inclusion of a polysaccharide fraction to account for certain of its histological properties.

In the questions dealt with above concerning the involvement of polysaccharide in the stabilization of the elastin fibre, the tacit [illegible] portion of the fibre con- [illegible] ined [illegible] one another in the fibrous phase. This is in complete agreement with the earlier observations of Partridge and co-workers (1951) but the observations of Lansing, Roberts, Ramasarma, Rosenthal and Alex (1951) cannot be correlated with this concept since they call for the existence of elastins having different amino-acid compositions in samples of elastic tissue from different age groups. A possible explanation of this has been afforded by the observations of Hall, Keech, Reed, Saxl, Tunbridge and Wood who suggested the possibility that their *in vitro* observations on the conversion of collagen to elastin could be extrapolated to cover the *in vivo* field (1955). Degeneration of collagen fibres prior to the synthesis of elastin fibres would explain why various elastin preparations appear to contain amino acids over and above those normally associated with the classical elastin analyses since the breakdown of collagen from the older age groups is not so complete and collagen fibres which have not degraded to the extent necessary for the production of classical elastin will become embodied in the forming elastic fibre. Some of the collagen fibres do apparently, however, convert completely to elastin, since Hall has shown (1951) that the extraction

with urea solution of aortic elastin preparations which have an amino-acid pattern between that of collagen and elastin produces a product in the penultimate stage before dissolution having an amino-acid composition in close conformity to that of classical elastin.

It appears, therefore, as if there may not be a single entity 'elastin'

A model for the structure of elastin based on the incomplete evidence as yet at our disposal must of necessity be rather tentative, but

sidered as consisting of a two-phase system, both outer and inner phases being composed of identical protein units, the outer ones being linked through a small amount of polysaccharide, whereas the

The protein unit would be identifiable as the β-fraction, having a molecular weight of 5500 whereas the major α-fraction would represent linear polymers of this smaller sub-unit linked by bonds

autoclaving removes such a high proportion of the polysaccharide. Secondly, Partridge has noted that the β-fraction is released preferentially in the earlier stages of attack, whereas the α-protein is liberated preferentially only in the later stages of the reaction. These observations are easily explicable if one accepts the hypothesis that the β-fraction represents a portion of the outer sheath, while the α-fraction is the substance of the inner fibrous core.

The author wishes to acknowledge the active help and sustained interest of Professor R. E. Tunbridge. He also wishes to thank Dr. G. C. Wood and Miss H. Saxl for much helpful discussion.

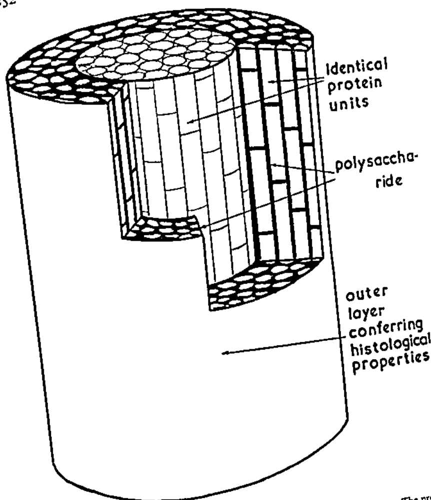


Fig 5
An idealized representation of the author's suggested model for an elastin fibre The protein units making up the whole of the inner core and the majority of the outer core are identical and are directly linked in the former phase, but are linked through polysaccharide in the latter phase

GROUP DISCUSSION

In reply to Dr. Snellman, Dr. Hall said that it was possible to absorb E_1 on to the surface of elastin but he did not know whether it could be precipitated by means of a polysaccharide.

Dr. Sylvén asked Dr. Partridge if his fractions were tested by the usual staining reactions for elastin, collagen and others, and Dr. Partridge replied that he had not investigated the staining reactions of the soluble materials. His purified fibres, however, were stained with orcein like elastic tissue, and were not stained by basic and acidic dyes.

[illegible]

specifically strip off the outer coating to see if at the same time this removed staining properties Dr. Hall replied that this was just what was observed when elastase was used.

Dr. Meyer said the purified polysaccharides which he had isolated from *ligamentum nuchae* are not acted upon by crude elastase prepared by Banga's procedure or by a mould enzyme mixture containing elastase but that he did not claim to have isolated all the polysaccharides present in *ligamentum nuchae*.

Referring to the insolubility of the polysaccharide described by Dr. Hall, he said that insolubility was characteristic of mucoids extracted from many tissues and was probably due to denaturation He referred to the unreliability of reducing value measurements on hydrolysates of mucopolysaccharides as a measure of their reducing sugar content

Dr. Hall replied that measurements of reducing power had been made only to show that the polysaccharides isolated from *ligamentum nuchae* did not contain CSA. Dr Meyer did not think this conclusion was

[illegible]

that E, is necessarily a polysaccharide-splitting enzyme. He was prepared to accept the idea that the elastin fibres are coated by a polysaccharide protein complex but did not think that the nature of the protein component was yet settled

Th [illegible]
that t [illegible]
terms [illegible]
they appear in the tissue

THE STRUCTURE AND CHEMICAL COMPOSITION OF CONNECTIVE TISSUE

I. Banga and J. Baló

Connective tissue fibres showing homogeneity under the light

the normal. No fibre is capable of functioning unless its chemical structure is intact.

The present work deals with the mucoprotein components of connective tissue fibres and with those enzymes which take part in

and mucopoly-

only a small part

of these – greatly influence the physico-chemical behaviour, swelling, permeability and functional capability of the fibres. Our experiments show that some colloidal properties of the fibres, for example the acid and neutral swelling, seem to be related not only to the electrovalent or hydrogen bonds of the polypeptide chains but also to the cross linkages present between the mucoprotein and polypeptide chains. We believe that although the mucopolysaccharides form only a small part of the connective tissue fibre nevertheless their biological importance is very great.

ENZYMES CAPABLE OF DEGRADING CONNECTIVE TISSUE: ELASTASE, *MUCOPROTEINASES AND HYALURONIDASE*

connective tissue

are

he

fibres Through these agents the fibres can be decomposed gradually and the split products can be analysed separately.

Among the specific enzymes we would like to mention elastase (Baló and Banga, 1949, 1950) which was a very valuable agent in the investigation of the structure of connective tissue fibres on [illegible]

a proteolytic enzyme. We want to maintain the name elastase for this proteolytic enzyme mainly because we succeeded recently in isolating and crystallizing an enzyme which dissolves elastin and [illegible]

orated this suggestion (Banga and Baló, 1956) by isolating from the pancreas two mucoproteinases which split specifically the mucoproteins bound to connective tissue but which do not digest the polypeptide chain. These mucoproteinases usually occur as contaminants in the elastase preparations.

The mucolytic enzymes of the pancreas are not homogeneous So far we have shown by preparative methods as well as by electrophoretic examinations and by specific activity measurements on different substrates that there are at least three different mucoproteinases. One of these shows its greatest specific activity on the elastomucin component of elastic fibre; therefore we have termed it elastomucoproteinase. The specific substrate for elastomucoproteinase seems to be identical with the alkali-labile component of elastic fibre.

The second enzyme splits one mucoprotein component of collagen fibre, therefore it is called collagen-mucoproteinase. The third enzyme shows a specificity to the mucoprotein component of the blood serum· we call this enzyme serum-mucoproteinase (Banga, 1955). This group of mucolytic enzymes differs from the hyaluronidases and from the other known enzymes of the pancreas and represents a new group of enzymes. The isolation of mucoproteinase is in progress. So far we have only obtained one mucolytic enzyme in pure crystalline form and as an electrophoretically homogeneous protein. This is the collagen-mucoproteinase.

Besides elastase and mucoproteinases we have been able to demonstrate the activity of Hyason (Organon) on the collagen fibre. In this case a preliminary treatment with mucoproteinase is necessary.

THE STRUCTURE AND CHEMICAL COMPOSITION OF CONNECTIVE TISSUE

I. BANGA AND J. BALÓ

Connective tissue fibres showing homogeneity under the light [illegible]

are loosened or broken, the fibre will show altered function and the physico-chemical properties of the fibre will also be different from the normal. No fibre is capable of functioning unless its chemical structure is intact.

The present work deals with the mucoprotein components of connective tissue fibres and with those enzymes which take part in the degradation of the fibre. The mucoproteins and mucopolysaccharides bound to the fibres – although forming only a small part of these – greatly [illegible] ing, permeability [illegible] experiments show [illegible] example the acid and neutral swelling, seem to be related not only to the electrovalent or hydrogen bonds of the polypeptide chains but [illegible]

their biological importance is very great.

ENZYMES CAPABLE OF DEGRADING CONNECTIVE TISSUE: ELASTASE, MUCOPROTEINASES AND HYALURONIDASE

The investigations of the complex structure of connective tissue fibres have become only recently possible now that some agents are at our disposal which act specifically upon some ingredients of the fibres. Through these agents the fibres can be decomposed gradually and the split products can be analysed separately.

Among the specific enzymes we would like to mention elastase (Baló and Banga, 1949, 1950) which was a very valuable agent in

[illegible]

shown that one of the components of the elastase enzyme complex is a proteolytic enzyme. We want to maintain the name elastase for this proteolytic enzyme mainly because we succeeded recently in isolating and crystallizing an enzyme which dissolves elastin and [illegible]

[illegible]

orated this suggestion (Banga and Baló, 1956) by isolating from the pancreas two mucoproteinases which split specifically the mucoproteins bound to connective tissue but which do not digest the polypeptide chain. These mucoproteinases usually occur as contaminants in the elastase preparations

The mucolytic enzymes of the pancreas are not homogeneous. So far we have shown by preparative methods as well as by electrophoretic examinations and by specific activity measurements on different substrates that there are at least three different mucoproteinases. One of these shows its greatest specific activity on the elastomucin component of elastic fibre; therefore we have termed it elastomucoproteinase. The specific substrate for elastomucoproteinase seems to be identical with the alkali-labile component of elastic fibre.

The second enzyme splits one mucoprotein component of collagen fibre, therefore it is called collagen-mucoproteinase. The third enzyme shows a specificity to the mucoprotein component of the blood serum: we call this enzyme serum-mucoproteinase (Banga, 1955). This group of mucolytic enzymes differs from the hyaluronidases and from the other known enzymes of the pancreas and represents a new group of enzymes. The isolation of mucoproteinase is in progress. So far we have only obtained one mucolytic enzyme in pure crystalline form and as an electrophoretically homogeneous protein. This is the collagen-mucoproteinase.

Besides elastase and mucoproteinases we have been able to demonstrate the activity of Hyason (Organon) on the collagen fibre. In this case a preliminary treatment with mucoproteinase is necessary.

THE STRUCTURE AND CHEMICAL COMPOSITION OF CONNECTIVE TISSUE

I. BANGA AND J. BALÓ

Connective tissue fibres showing homogeneity under the light

true chemical bonds or by van der Waals forces. If some of the bonds are loosened or broken, the fibre will show altered function and the physico-chemical properties of the fibre will also be different from the normal. No fibre is capable of functioning unless its chemical structure is intact.

The present work deals with the mucoprotein components of connective tissue fibres and with those enzymes which take part in the degradation of the fibre. The mucoproteins and mucopolysaccharides bound to the fibres – although forming only a small part of these – greatly influence the physico-chemical behaviour, swelling, permeability and functional capability of the fibres. Our experiments show that some colloidal properties of the fibres, for example the acid and neutral swelling, seem to be related not only to the electrovalent or hydrogen bonds of the polypeptide chains but also to the cross linkages present between the mucoprotein and poly-

les

ess

their biological importance is very great.

ENZYMES CAPABLE OF DEGRADING CONNECTIVE TISSUE: ELASTASE, MUCOPROTEINASES AND HYALURONIDASE

connective tissue

Among the specific enzymes we would like to mention elastase (Baló and Banga, 1949, 1950) which was a very valuable agent in [illegible] shown that one of the components of the elastase enzyme complex is a proteolytic enzyme. We want to maintain the name elastase for this proteolytic enzyme mainly because we succeeded recently in isolating and crystallizing an enzyme which dissolves elastin and which was proved to be homogeneous by electrophoresis.

Hall, Reed and Tunbridge (1952) first described mucolytic properties of elastase. Hall (1953) produced evidence that elastase contains two enzymes one of which must be mucolytic. We corroborated this suggestion (Banga and Baló, 1956) by isolating from the pancreas two mucoproteinases which split specifically the mucoproteins bound to connective tissue but which do not digest the polypeptide chain. These mucoproteinases usually occur as contaminants in the elastase preparations.

The mucolytic enzymes of the pancreas are not homogeneous. So far we have shown by preparative methods as well as by electrophoretic examinations and by specific activity measurements on different substrates that there are at least three different mucoproteinases. One of these shows its greatest specific activity on the elastomucin component of elastic fibre; therefore we have termed it elastomucoproteinase. The specific substrate for elastomucoproteinase seems to be identical with the alkali-labile component of elastic fibre.

The second enzyme splits one mucoprotein component of collagen fibre, therefore it is called collagen-mucoproteinase The third enzyme shows a specificity to the mucoprotein component of the blood serum: we call this enzyme serum-mucoproteinase (Banga, 1955). This group of mucolytic enzymes differs from the hyaluronidases and from the other known enzymes of the pancreas and represents a new group of enzymes. The isolation of mucoproteinase is in progress. So far we have only obtained one mucolytic enzyme in pure crystalline form and as an electrophoretically homogeneous protein. This is the collagen-mucoproteinase.

Besides elastase and mucoproteinases we have been able to demonstrate the activity of *Hyason (Organon) on the collagen fibre.* In this case a preliminary treatment with mucoproteinase is necessary.

THE STRUCTURE AND CHEMICAL COMPOSITION OF CONNECTIVE TISSUE

I. Banga and J. Baló

Connective tissue fibres showing homogeneity under the light microscope and homogeneity by histological standards represent a chemically complex system containing many components. More-

are loosened or broken, the fibre will show altered function and the physico-chemical properties of the fibre will also be different from the normal. No fibre is capable of functioning unless its chemical structure is intact.

The present work deals with the mucoprotein components of connective tissue fibres and with those enzymes which take part in the degradation of the fibre The mucoproteins and mucopolysaccharides bound to the fibres – although forming only a small part of these – greatly influence the physico-chemical behaviour, swelling, *permeability and functional capability of the fibres.* Our experiments show that some colloidal properties of the fibres, for example the acid and neutral swelling, seem to be related not only to the electrovalent or hydrogen bonds of the polypeptide chains but

their biological importance is very great.

ENZYMES CAPABLE OF DEGRADING CONNECTIVE TISSUE: ELASTASE, MUCOPROTEINASES AND HYALURONIDASE

The investigations of the complex structure of connective tissue fibres have become only recently possible now that some agents are at our disposal which act specifically upon some ingredients of the fibres. Through these agents the fibres can be decomposed gradually and the split products can be analysed separately.

Among the specific enzymes we would like to mention elastase (Baló and Banga, 1949, 1950) which was a very valuable agent in the investigation of the structure of connective tissue fibres on account of its properties of dissolving elastic and metacollagen fibres (Banga, Baló and Szabó, 1956). Partridge and Davis (1955) have shown that one of the components of the elastase enzyme complex is a proteolytic enzyme. We want to maintain the name elastase for this proteolytic enzyme mainly because we succeeded recently in isolating and crystallizing an enzyme which dissolves elastin and

orated this suggestion (Banga and Baló, 1956) by isolating from the pancreas two mucoproteinases which split specifically the mucoproteins bound to connective tissue but which do not digest the polypeptide chain. These mucoproteinases usually occur as contaminants in the elastase preparations.

The mucolytic enzymes of the pancreas are not homogeneous. So far we have shown by preparative methods as well as by electrophoretic examinations and by specific activity measurements on different substrates that there are at least three different mucoproteinases. One of these shows its greatest specific activity on the elastomucin component of elastic fibre; therefore we have termed it elastomucoproteinase. The specific substrate for elastomucoproteinase seems to be identical with the alkali-labile component of elastic fibre.

The second enzyme splits one mucoprotein component of collagen fibre, therefore it is called collagen-mucoproteinase. The third enzyme shows a specificity to the mucoprotein component of the blood serum· we call this enzyme serum-mucoproteinase (Banga, 1955) This group of mucolytic enzymes differs from the hyaluronidases and from the other known enzymes of the pancreas and represents a new group of enzymes. The isolation of mucoproteinase is in progress. So far we have only obtained one mucolytic enzyme in pure crystalline form and as an electrophoretically homogeneous protein. This is the collagen-mucoproteinase.

Besides elastase and mucoproteinases we have been able to demonstrate the activity of Hyason (Organon) on the collagen fibre. In this case a preliminary treatment with mucoproteinase is necessary

DIFFICULTY OF MEASURING THE ACTIVITY OF MUCOPROTEINASES

In connection with the examination of mucoproteinases there are two kinds of difficulties. The first one is the question of mucoproteins as substrates. In connective tissue the polypeptide chains make up 95 per cent of the fibre and the amounts of mucoproteins are only 3-5 per cent. These mucoproteins cannot be liberated by means of chemical methods without causing an alteration in their molecules. Therefore one is obliged to measure the mucoproteinase activity on entire fibres, that is on a substrate in which 95 per cent of the substance is useless and the specific substrate is present only in a small proportion. In consequence, some of the classical enzyme methods, for example the determination of the substrate concentration curve, cannot be carried out.

Furthermore, even the small amount of mucoprotein associated with any single connective tissue fibre cannot be considered homogeneous. The elastic fibre as well as the collagen fibre contains two or three different mucoprotein or mucopolysaccharide components.

The second difficulty consists in the fact that there are no methods whereby the amount of a single mucoprotein associated with the fibre can be measured quantitatively. The sensitivity ... methods for determination of ... is ins...
use ...
nec ...
... practicable. In order to overcome this difficulty ... the mucoproteins of connective tissue we have applied for our purpose the quantitative Molisch reaction of Száva (1956) (Banga and Baló, 1956). 1-Naphthol-2-sulphonic acid is water-soluble and gives different colour reactions with the various mucoproteins. We measure, therefore, the light absorption of the coloured solution at several ... mμ with the ... The colour ... mucoproteins ... On the basis of these experiments it seems that the ... is specific for the single mucoproteins of elastic and collagen fibres differ from one another. In consequence probably the enzymes are also different.

PROPERTIES OF MUCOPROTEINASE ENZYMES OF THE CONNECTIVE TISSUE FIBRES

According to the difficulties mentioned above we know ver little about the properties of the mucoproteinases of connectiv tissue. Table I summarizes the present data relating to the muco-

TABLE I

Enzymes	MUCOPROTEINASES Substrate	Stability	End-products
Elasto-Mucoproteinase	Cervical Ligament (Collagen-free) Aorta (Collagen-free)	2 components Thermolabile + Thermostable	2 components Undialysable Mucoprotein (75 per cent) + Dialysable sugar (25 per cent)
Collagen-Mucoproteinase	Achilles Tendon Rat Tail Tendon Procollagen Metacollagen	2 components Thermolabile + Thermostable	2 components Undialysable Mucoprotein (75 per cent) + Dialysable Sugar (25 per cent)
Serum Mucoproteinase	Serum protein	Thermolabile	Mucoprotein Sugar?

proteinases isolated from bovine pancreas We call these enzymes mucoproteinases because they split off the fibres not only carbohydrates but also mucoproteins as judged by the appearance in solution of large amounts of undialysable nitrogen Thus nitrogen cannot be derived from hexosamine because the latter is present only in small quantity. The mucoproteinases split besides the mucoprotein components also a dialysable carbohydrate component containing nitrogen. The latter component is approximately 20–25 per cent of the components split by the enzymes Due to the small quantities split, no further analytical results are available up to now.

The data of Table I show that besides the thermolabile components both the elastomucoproteinase as well as collagen-mucoproteinase contain also heat-stable components. So far it is undecided whether these components found in both of the enzymes are identical or not.

THE DIFFICULTY OF MEASURING THE ACTIVITY OF MUCOPROTEINASES

In connection with the examination of mucoproteinases there are two kinds of difficulties. The first one is the question of mucoproteins as substrates. In connective tissue the polypeptide chains make up 95 per cent of the fibre and the amounts of mucoproteins are only 3-5 per cent. These mucoproteins cannot be liberated by means of chemical methods without causing an alteration in their molecules. Therefore one is obliged to measure the mucoproteinase activity on entire fibres, that is on a substrate in which 95 per cent of the substance is useless and the specific substrate is present only in a small proportion. In consequence, some of the classical enzyme methods, for example the determination of the substrate concentration curve, cannot be carried out.

Furthermore, even the small amount of mucoprotein associated [illegible]

The second difficulty consists in the fact that there are no methods [illegible]

is influenced by the presence of proteins to such an extent that their use for the quantitative measurement of the mucoproteins of connective tissue is impracticable. In order to overcome this difficulty [illegible]

of the coloured solution at several wave-lengths from 420 to 750 mμ with the Stuphophotometer (Zeiss) by the use of colour filters. The colour absorption curve thus obtained is specific for the single mucoprotein. On the basis of these experiments it seems that the mucoproteins of elastic and collagen fibres differ from one another. In consequence probably the enzymes are also different.

TABLE II

FRACTIONAL DEGRADATION OF COLLAGEN

Effects	*Substrate*	*Constituents*	*End-product*	*Constituents*	*Elastase solubility*	*Function capable of contraction relaxation*
(1) Heat effect of 40 per cent *KI*	Native Collagen Fibre of Rat *Tail tendon*	Procollagen Metacollagen Chondromucin (CHS B + C) Mucoprotein$_1$ Mucoprotein$_2$	Metacollagen (Elastic)	Metacollagen Mucoprotein$_2$	Soluble	No contraction (Syneresis)
(2) Mucoproteinase	"	"	Collagen Residuum	Procollagen Metacollagen CHS B + C	None	After contraction tears
(3) Hyason (Organon)	"	"	Collagen Residuum	Procollagen Metacollagen Mucoprotein$_1$ Mucoprotein$_2$ CHS B	None	Contraction—Relaxation remains unchanged
(4) Mucoproteinase (first) Hyason (after)	"	"	Collagen Residuum (elastic)	Procollagen Metacollagen CHS B	Degraded to filaments	No contraction
(5) Hyason (first) Mucoproteinase (after)	"	"	Collagen Residuum	Procollagen Metacollagen CHS B + C?	None	After contraction tears
(6) pH_4 Citrate Extraction and 1h 37° C H_2O	"	"	Collastromin	Metacollagen Mucoprotein$_2$ CHS B + C	None	No contraction

THE FRACTIONAL DECOMPOSITION OF MUCOPROTEINS, I.E. MUCOPOLYSACCHARIDES OF COLLAGEN FIBRES

Table II summarizes our results according to which the collagen fibre – as it has a complex constitution – yields different structures by applying different chemical substances or enzymes as degradation agents.

According to our still incomplete knowledge the native collagen fibres contain, besides procollagen and metacollagen, also chondromucines comprising the chondroitin sulphate B and C (CHS. B, C). Furthermore, it contains two other mucoproteins termed mucoprotein$_1$ and mucoprotein$_2$ (Banga and Baló, 1956).

Among the different effects on the collagen fibres (cf. Table II, 1) the effect of heat and that of 40 per cent KI solution will be discussed, *first as they cause the native collagen fibres to contract and relax*
(Banga, Baló
metacollagen
mucoprotein$_2$.
entirely by elastase.

As is shown in Table II, 2, the effect of mucoproteinases is to produce a kind of collagen fibre – called collagen residuum – which is not dissolved by elastase. It cannot do work connected with the contraction-relaxation phenomenon. It tears after contraction.

In Table II, 3, we describe the effect of hyason in the presence of physiological solution of NaCl and call the fibre again collagen residuum. In our opinion this fibre differs from the native fibre only by not containing CHS. C. This lack does not influence the stability of the fibre because *it is capable of showing the phenomenon* of contraction and relaxation. We should like to note that in the experiment described by Jackson (1954) the hyaluronidase was dissolved in acetate buffer of pH 5.2 containing 0.15 M NaCl which solution will dissolve the procollagen even without hyaluronidase and will cause a decrease in the stability of the fibre. Therefore in the experiments of Jackson two kinds of effect come into action: the effect of buffer which dissolves the procollagen and the effect of hyaluronidase which dissolves the CHS. C from the collagen fibre.

The experiments in Table II, 4 and 5 reveal interesting data. In the ground substance of tendon, CHS. *B and C and* hyaluronate are present (Meyer, 1954) and are dissolved by the action of hyaluronidase. Our experiments have shown that by avoiding the use of acid

strated in very old animals. The other difference manifests itself if the treatment shown in Table II, 4 and 5 is applied to the collagen fibres derived from animals of different ages. The effect of Hyason after the treatment by mucoproteinase releases components giving different colours with the Szára reagent. After mucoproteinase treatment, Hyason dissolves from young fibre, 2–3 weeks old, a component which gives with the Szára reagent a colour with a maximum absorption at 530 mμ. In old animals the same component has its maximum absorption at 570 mμ. These differences show the change with age in the quality of mucoproteins in the fibres.

DISCUSSION

During the last few years, the number of papers dealing with the association between polysaccharides, chondroitin sulphates and hyaluronates and the proteins of collagen have increased. We would particularly like to refer to the work of Highberger, Gross and Schmitt (1951, 1953, 1954) who in their electron microscopic studies produced evidence on the genesis of fibrils from acid soluble collagen. Delaunay, Bazin, Fauve and Henon (1956) proved experimentally the association between the mucopolysaccharides of umbilical cord and collagen and have suggested the existence of strong linkages These authors believe that these linkages play also an important role *in vivo*. Grassmann and Kühn (1955) have also shown that procollagen contains mucopolysaccharides. We could also demonstrate with the Szára reaction (unpublished data) that procollagen contains a large amount of mucopolysaccharides (6–8 per cent) which remains in the molecule after purification

In the present work we have given further evidence on the existence of specific enzymes acting on the mucoproteins of connective tissues and we think that the mucoproteins present in connective tissue fibres play an important role. Histologists have for a long time paid special attention to the metachromasia of connective tissues as well [illegible] con-
nec[illegible] ironi-
das[illegible] search
on mucopolysaccharides of connective tissue. However, most studies have so far been restricted to the ground substance. Our study attaches importance to the analysis of the fibre and endeavours
s[illegible]

buffer which dissolves the procollagen, as mentioned above, there i[illegible] very little polysaccharide which can be dissolved by Hyason. I[illegible] consequence, there is no change in function of the fibre. But if th[illegible] collagen fibre is previously treated with pancreatic mucoproteinas[illegible] and afterwards by Hyason (T.R. 2.5/ml.) then it can be shown tha[illegible] Hyason will also dissolve a mucoid that changes the stability of th[illegible] fibre. After the effect of both enzymes, a fibre remains which ha[illegible] elasticity confirming the views of Hall, Keech, Reed, Saxl, Tunbridge and Wood (1955) and of Burton, Hall, Keech, Reed, Saxl, Tunbrid[illegible]

elastin.

enzymes [illegible]

stances. This elastic collagen is not dissolved without residue by elastase but it disintegrates into filaments. If the order is changed and the fibre is treated first with Hyason and afterwards with mucoproteinase then the effect is different and is similar to that found when the fibres were treated with mucoproteinase alone. This experiment shows that Hyason can have a profound effect on the collagen fibre only if another mucoid has been dissolved previously. The dissolution of [illegible]

[illegible] *et al.* (1954), is obtained. The collastromin contains, besides a polypeptide chain, mucoproteins and CHS. A, B and C. Collastromin is insoluble in elastase and does not show heat or chemical contraction.

THE EFFECT OF AGE ON THE COMPOSITION OF MUCOPROTEINS OF COLLAGEN FIBRE

It has been shown that the mucoprotein composition of collagen fibres varies according to age. For the demonstration of the differences the Szára reaction mentioned above is especially suitable. The main qualitative difference between young and old fibres is revealed by the fact that from the collagen fibres of young animals mucoproteinase releases a substance which gives with the Szára reagent a red colour with green fluorescence. The fibres of old animals fail to show this fluorescence. The fluorescent component disappears step by step from the fibre with age and cannot be demon-

rate than the native serum, and thus she attributed to the presence of inhibitors in the serum which were removed by ethanol. In reply to Dr Snellman, DR BANGA said that that she had not yet isolated the acid mucoprotein from serum.

In reply to Dr Grassmann, DR. BANGA said that the collagen she had used was alcohol-dried Achilles tendon and also Achilles tendon which had been treated with sodium hydroxide. The resulting material contained 0 6 per cent to 0 9 per cent polysaccharide.

DR GRASSMANN said that he had always obtained similar figures for the carbohydrate content He then asked how many per cent of protein nitrogen were dissolved from the collagen and what was the relation between the sugars and the nitrogen in the dialysable part.

DR. BANGA answered that she determined only the polysaccharide of the dialysable and the undialysable part

There was some discussion between Dr Grassmann and Dr. Meyer regarding the reliability of methods for estimating sugars in hydrolysates of mixtures of polysaccharide and protein. DR GRASSMANN said that a more reliable estimation could be made by using mild hydrolysis conditions and separating the amino acids from the sugars as their DNP derivatives by column chromatography. Dr Meyer was sceptical.

to establish conditions for examining the mucoproteins bound to the polypeptide chain of the fibres. Through the possession of pancreatic mucoproteinases we hope to gain a clearer picture of the relation between the constituents of the connective tissue fibre.

SUMMARY

(1) Bovine pancreas contains, in addition to elastase which has proteolytic activity on connective tissue fibres, mucolytic enzymes as well which act on the mucoproteins. These enzymes can be looked upon as a new group of enzymes which differ from the known pancreatic enzymes and seem to be specific for the mucoproteins of connective [illegible]

[illegible]

collagen mucoproteinase and serum mucoproteinase respectively.

(2) The difficulties of examining mucoproteins and mucopolysaccharides have been discussed and a method was described for their examination.

(3) The mucoproteins bound to the fibres were shown to vary qualitatively according to the age of the animals.

(4) The gradual enzymic release of the different mucoproteins from collagen fibres leads to an alteration of their function.

GROUP DISCUSSION

In reply to Dr. Orekhovitch, DR. BANGA said that procollagen and metacollagen are not identical but that metacollagen resembles the collastromin of Tustanowski. However, unlike metacollagen, collastromin is not dissolved by elastase.

Answering Dr. Meyer, DR. BANGA said that in addition to the Molisch and the orcein methods she had used the anthrone method to determine [illegible]

she had used came from the pancreas.

In reply to Dr. Me[illegible]

mucoproteinase, the [illegible]

substrate was not isol[illegible]

The ethanol precipitated serum which could be looked upon as a denatured substrate was also attacked by the enzyme and, indeed, at a greater

rate than the native serum, and this she attributed to the presence of inhibitors in the serum which were removed by ethanol. In reply to Dr. Snellman, DR. BANGA said that that she had not yet isolated the acid mucoprotein from serum.

[illegible]

DR GRASSMANN said that he had always obtained similar figures for the carbohydrate content. He then asked how many per cent of protein [illegible]

There was some discussion between Dr Grassmann and Dr. Meyer regarding the reliability of methods for estimating sugars in hydrolysates of mixtures of polysaccharide and protein. DR GRASSMANN said that a more reliable estimation could be made by using mild hydrolysis conditions and separating the amino acids from the sugars as their DNP derivatives by column chromatography Dr Meyer was sceptical.

THE COMPOSITION OF SOME PROTEIN FRACTIONS ISOLATED FROM BOVINE SKIN

J. H. Bowes, R. G. Elliott and J. A. Moss

Investigations by various groups of workers have shown that the collagen of the skin and other tissues can be subdivided into a number of different fractions of varying solubility, while evidence for the presence of one or more non-collagenous type proteins closely associated with the collagen is accumulating. Eastoe and Eastoe (1954) have demonstrated the presence of a protein, similar in composition to the serum proteins, in the mucopolysaccharide fraction isolated from bone collagen. The work of Consden and his collaborators (Consden, 1953; Consden, Glynn and Stanier, 1953) indicates the presence of a similar constituent in connective tissue and rabbit skin. It seems probable that this protein-polysaccharide fraction represents the main constituent of the ground substance.

During recent years the work in these laboratories has been concerned with the separation of various constituents from animal skin and determination of their chemical composition. Interest has primarily been centred on those constituents which are extracted from skin by dilute acid and alkaline solutions, and this paper summarizes the results of these investigations, and discusses their significance in relation to the structure of collagenous tissue.

The work has been mainly carried out on the skins of the larger [illegible]

fore, a matter of conjecture.

ACID-SOLUBLE COLLAGEN

Acid-soluble collagen was prepared from the skin of a 6- to 8-week-old bull calf as described by Orekhovich, Tustanowski, Orekhovich and Plotnikova (1948). The precipitate was purified

by redissolving in citrate buffer and dialysing against tap water. The amino-acid composition of the purified protein determined by the method of Moore and Stein (1951) is given in Table I, together

TABLE I

AMINO-ACID CONTENT OF OX-HIDE COLLAGEN AND OF CITRATE-SOLUBLE COLLAGEN OF CALF SKIN (FROM BOWES, ELLIOTT AND MOSS, 1955)

	Citrate-soluble protein			*Ox-hide collagen*		
	N as per cent protein-N	*gm/100 gm*	*gm residues/100 gm*	*N as per cent protein-N*	*gm/100 gm*	*gm residues/100 gm*
Total N	—	17.70	—	—	18.60	—
Amino N	2.65	0.49	—	2.50	0.46	—
Glycine	27.48	26.07	19.81	26.66	26.57	20.20
Alanine	8.84	9.95	7.94	8.72	10.32	8.23
Leucine	1.93	3.20	2.76	2.14	3.73	3.22
Isoleucine	0.84	1.39	1.20	1.08	1.88	1.62
Valine	1.53	2.26	1.91	1.58	2.46	2.08
Phenylalanine	0.95	1.98	1.78	1.07	2.35	1.95
Tyrosine	0.22	0.50	0.45	0.41	0.99	0.89
Tryptophan	—	—	—	—	—	—
Serine	3.19	4.23	3.51	3.06	4.27	3.54
Threonine	1.47	2.21	1.87	1.43	2.26	1.92
Cystine	—	—	—	—	—	—
Methionine	0.41	0.78	0.68	0.49	0.97	0.85
Proline	8.95	13.02	10.98	9.43	14.42	12.16
Hydroxyproline	8.22	13.62	11.75	7.37	12.83	11.07
Arginine	15.16	8.34	7.48	14.22	8.22	7.37
Histidine	0.45	0.29	0.26	1.02	0.70	0.62
Hydroxylysine	0.88	0.90	0.80	0.93	1.00	0.89
allo Hydroxylysine*	0.38	0.39	0.34	0.14	0.15	0.13
Lysine	3.87	3.57	3.13	4.08	3.96	3.47
Aspartic acid	3.60	6.05	5.23	3.93	6.95	6.01
Glutamic acid	5.93	11.02	9.69	5.69	11.16	9.75
Amide	2.92	0.52	—	3.50	0.66	—
Hexosamine	—	0.01	—	—	0.05	—
Total	97.30	—	91.56	96.97	—	95.97
Average residue weight						
By summation	—	—	90.5	—	90.9	—
By N-distribution	—	—	95.5	—	91.2	—

* Not identified, but assumed to be *allo*hydroxylysine for purposes of calculation

The nitrogen content of the citrate-soluble collagen was appreciably lower than that of the ox-hide collagen and it was, therefore, considered preferable to compare the composition of the two proteins on the basis of amino-acid nitrogen as a percentage of the total nitrogen, rather than as weight of amino acid per hundred parts of protein.

The composition of the two proteins is essentially the same, but there are a number of small differences. The amide, tyrosine and

Table II

TERMINAL RESIDUES IN CITRATE-SOLUBLE COLLAGEN (FROM BOWES, ELLIOTT AND MOSS, 1955)

	Citrate-soluble collagen (m-moles/100gm	Gelatin from citrate-soluble collagen DNP—protein)
Aspartic acid	0.06	0.14
Alanine	0.04	0.20
Glycine	—	0.14
Glutamic acid	—	0.17
Serine	—	Trace
Threonine	—	Trace

About 97 per cent of the total nitrogen is accounted for with both proteins, but, whereas with the ox-hide collagen the sum of the amino-acid residues adds up to 96 and the deficit could be due to overall losses of amino acids during analysis, with the citrate-soluble collagen the sum of the amino-acid residues is only 91.6. Even if all the nitrogen unaccounted for was present in amino acids of high molecular weight, the total of the residue weights would still fall appreciably short of a hundred. This, together with its low nitrogen content compared with ox-hide collagen, suggests the presence of about 4 to 5 per cent of some non-protein constituent of low nitro- ... failed.
s paper
nounts

ployed by Windrum *et al.* (1955) indicated the presence of less than 0.1 per cent of fat.

(Bowes and Moss, 1953). It is, perhaps, of interest that the N-terminal residues found in ox-hide collagen after treatment with hyaluronidase were the same as those found in the citrate-soluble collagen (Bowes and Moss, 1953). Unfortunately it is not certain that the hyaluronidase preparation used was completely free from proteolytic activity so that release of these end groups cannot with certainty be ascribed to the removal of polysaccharide. On warming to 40° C for a few minutes, further end groups were released from the citrate-soluble collagen and on cooling the solution set to a gel.

It is possible that the differences in the composition of ox-hide collagen and the citrate-soluble collagen found in this investigation

adult collagen, of a protein constituent which is relatively rich in amide-nitrogen, tyrosine, histidine and to a less extent leucine, isoleucine and possibly aspartic acid, and low in hydroxyproline compared with the citrate-soluble collagen.

Some preliminary experiments on the extraction of tendon with citrate buffer suggested that a protein fraction of low hydroxy-

The nitrogen content of the citrate-soluble collagen was appreciably lower than that of the ox-hide collagen and it was, therefore, considered preferable to compare the composition of the two proteins on the basis of amino-acid nitrogen as a percentage of the total nitrogen, rather than as weight of amino acid per hundred parts of protein.

The composition of the two proteins is essentially the same, but there are a number of small differences. The amide, tyrosine and histidine contents of the citrate-soluble collagen are definitely lower, and the leucine, isoleucine, and possibly aspartic acid, contents are [illegible]

TABLE II

TERMINAL RESIDUES IN CITRATE-SOLUBLE COLLAGEN (FROM BOWES, ELLIOTT AND MOSS, 1955)

	Citrate-soluble collagen (m-moles/100gm	*Gelatin from citrate-soluble collagen DNP—protein)*
Aspartic acid	0 06	0 14
Alanine	0 04	0 20
Glycine	—	0 14
Glutamic acid	—	0 17
Serine	—	Trace
Threonine	—	Trace

About 97 per cent of the total nitrogen is accounted for with both proteins, but, whereas with the ox-hide collagen the sum of the amino-acid residues adds up to 96 and the deficit could be due to overall losses of amino acids during analysis, with the citrate-soluble collagen the sum of the amino-acid residues is only 91.6. Even if all the nitrogen unaccounted for was present in amino acids of high molecular weight, the total of the residue weights would still fall appreciably short of a hundred. This, together with its low nitrogen content compared with ox-hide collagen, suggests the presence of about 4 to 5 per cent of some non-protein constituent of low nitrogen content. Attempts to detect such a constituent have so far failed. Determination of reducing sugars and hexosamine, as well as paper chromatography, indicate the presence of only very small amounts

with phosphate buffer, 20 per cent with citrate buffer, another 8.5 per cent with dilute acetic acid, and 2.2 per cent with dilute alkali, leaving a residue of about 60 per cent. In the citrate buffer and acetic acid extracts nearly all the protein was precipitated by sodium chloride leaving only very small amounts of nitrogen in solution, representing about 0.3 and 0.2 per cent of the total-nitrogen respectively. Of this material remaining in solution only about one-quarter was recovered after dialysis and concentration. In the alkaline extracts about half the nitrogen was precipitated by alcohol.

Amino Acid Composition of Protein Fractions

The hydroxyproline and tyrosine contents of some of the fractions were determined in order to gain information as to their identity (see Table III). The method of Neuman and Logan (1950) was used

TABLE III

HYDROXYPROLINE AND TYROSINE CONTENT OF SKIN FRACTIONS

	Hydroxyproline-N	*Tyrosine-N*	*Collagen-N**
	Amino or Imino-acid-N as per cent Total-N		
Citrate-Soluble Proteins			
Precipitated by NaCl			
Extract 1	7.68	0.32	93.4
2	7.95	0.19	97.0
4	7.88	0.29	95.8
7	8.15	0.26	99.3
Not precipitated by NaCl	0.1	0.56	1.2
Acetic Acid-Soluble Proteins			
Precipitated by NaCl			
Extract 2	7.47	0.30	90.6
4	7.73	0.28	94.2
6	7.86	0.27	93.8
7	7.66	0.30	93.3
10	7.48	0.31	90.4
Not precipitated by NaCl	0.19	1.67	2.3
Alkali-Soluble Protein			
Precipitated by ethanol			
Extract 2	0.42	2.33	5.1
4	0.93	0.91	11.3
5	1.51	1.08	17.7
6	1.58	1.54	19.3
Residue after extraction	7.62	0.31	92.9

* Calculated from the hydroxyproline content assuming a value of 8.22 for hydroxyproline-N expressed as a per cent of total collagen-N

further this, and other fractions extracted from calf skin by dilute acid and alkaline solutions.

EXTRACTION OF CALF SKIN WITH DILUTE ACID AND ALKALINE SOLUTIONS

Extraction Procedure

A fresh skin from a bull calf about 5 weeks old was used for this [illegible] contamina- [illegible] nds [illegible] was used. This was obtained by sectioning 1-inch punches on a freezing microtome. The first few sections from the flesh side were discarded and the following sections collected until the base of the hair follicles was reached. The sections were transferred immediately to 0.1 *M* disodium hydrogen phosphate solution, pH 9.1 and stored below 4° C. In all about 470 punches were sectioned and the wet [illegible] The sections [illegible] s their [illegible] as pos- [illegible] tained

sible, the plasma proteins. The final phosphate [illegible] less than 0 02 mg. nitrogen per ml. corresponding to less than 0 1 per cent of the total-nitrogen of the skin. The macerate was then extracted seven times with four times its weight of 0 1 *M* citrate buffer, pH 3.7, until the nitrogen content of the extracts again fell to a low value (0 07 mg. N/ml.). The citrate buffer was followed by ten extractions with 0.05 *M* acetic acid adjusted to pH 2.8 with hydrochloric acid. The macerate was finally neutralized, and extracted seven times with sodium hydroxide solution, pH 12.0 to 12.3. All extractions were carried out at 0° to 4° C, and were, in general, each of 24 hours' duration. The acid-soluble collagen was precipitated from the extracts by the addition of sodium chloride to a final concentration of 5 per cent. The filtrates from the citrate-buffer extracts, with the exception of the first, were combined, [illegible] salts and concentrated The concentration was [illegible]

a stream of [illegible]

solutions were treated similarly. The alkaline extracts were

ized, concentrated and precipitated with three volumes of ethanol

About 8.5 per cent of the total-nitrogen of the skin was extracted

with phosphate buffer, 20 per cent with citrate buffer, another 8.5 per cent with dilute acetic acid, and 2.2 per cent with dilute alkali, leaving a residue of about 60 per cent. In the citrate buffer and acetic acid extracts nearly all the protein was precipitated by sodium chloride leaving only very small amounts of nitrogen in solution, representing about 0.3 and 0.2 per cent of the total-nitrogen respectively. Of this material remaining in solution only about one-quarter was recovered after dialysis and concentration. In the alkaline extracts about half the nitrogen was precipitated by alcohol.

Amino Acid Composition of Protein Fractions

The hydroxyproline and tyrosine contents of some of the fractions were determined in order to gain information as to their identity (see Table III). The method of Neuman and Logan (1950) was used

TABLE III

HYDROXYPROLINE AND TYROSINE CONTENT OF SKIN FRACTIONS

	Hydroxyproline-N	*Tyrosine-N*	*Collagen-N**
	Amino or Imino-acid-N as per cent Total-N		
Citrate-Soluble Proteins			
Precipitated by NaCl			
Extract 1	7.68	0.32	93.4
2	7.95	0.19	97.0
4	7.88	0.29	95.8
7	8.15	0.26	99.3
Not precipitated by NaCl	0.1	0.56	1.2
Acetic Acid-Soluble Proteins			
Precipitated by NaCl			
Extract 2	7.47	0.30	90.6
4	7.73	0.28	94.2
6	7.86	0.27	93.8
7	7.66	0.30	93.3
10	7.48	0.31	90.4
Not precipitated by NaCl	0.19	1.67	2.3
Alkali-Soluble Protein			
Precipitated by ethanol			
Extract 2	0.42	2.33	5.1
4	0.93	0.91	11.3
5	1.51	1.08	17.7
6	1.58	1.54	19.3
Residue after extraction	7.62	0.31	92.9

* Calculated from the hydroxyproline content assuming a value of 8.22 for hydroxyproline–N expressed as a per cent of total collagen-N

further this, and other fractions extracted from calf skin by dilute acid and alkaline solutions.

EXTRACTION OF CALF SKIN WITH DILUTE ACID AND ALKALINE SOLUTIONS

Extraction Procedure

A fresh skin from a bull calf about 5 weeks old was used for this investigation. In order to reduce complications due to contamination with keratinous material, elastin, muscle fibres, sebaceous glands and other cellular material, only the middle layer of the skin was [illegible]

follicles was reached. The sections were transferred immediately to 0.1 M disodium hydrogen phosphate solution, pH 9.1 and stored below 4° C. In all about 470 punches were sectioned and the wet weight of the sectioned skin obtained was about 400 gm. The sections of skin were extracted five times with approximately five times their weight of 0.1 M phosphate buffer, pH 9.1, to remove, as far as possible, the plasma proteins. The final phosphate extract contained [illegible]

extracted seven times with four times its weight of [illegible] buffer, pH 3.7, until the nitrogen content of the extracts again fell to a low value (0.07 mg. N/ml.). The citrate buffer was followed by ten extractions with 0.05 M acetic acid adjusted to pH 2.8 with hydrochloric acid. The macerate was finally neutralized, and extracted seven times with sodium hydroxide solution, pH 12.0 to 12.3. All extractions were carried out at 0° to 4° C, and were, in general, each of 24 hours' duration. The acid-soluble collagen was precipitated from the extracts by the addition of sodium chloride to a final concentration of 5 per cent. The filtrates from the citrate-buffer extracts, with the exception of the first, were combined, [illegible] and concentrated. The concentration was [illegible]

a stream [illegible] solutions were treated similarly. The alkaline [illegible] ized, concentrated and precipitated with three volumes of ethanol.

About 8.5 per cent of the total-nitrogen of the skin was extracted

with phosphate buffer, 20 per cent with citrate buffer, another 8.5 per cent with dilute acetic acid, and 2.2 per cent with dilute alkali, leaving a residue of about 60 per cent In the citrate buffer and acetic acid extracts nearly all the protein was precipitated by sodium chloride leaving only very small amounts of nitrogen in solution, repre- [illegible]

about half the nitrogen was precipitated by alcohol.

Amino Acid Composition of Protein Fractions

[illegible]

TABLE III

HYDROXYPROLINE AND TYROSINE CONTENT OF SKIN FRACTIONS

	Hydroxyproline-N	*Tyrosine-N*	*Collagen-N**
	Amino or Imino-acid-N as per cent Total-N		
Citrate-Soluble Proteins			
Precipitated by NaCl			
Extract 1	7.68	0.32	93.4
2	7.95	0.19	97.0
4	7.88	0.29	95.8
7	8.15	0.26	99.3
Not precipitated by NaCl	0.1	0.56	1.2
Acetic Acid-Soluble Proteins			
Precipitated by NaCl			
Extract 2	7.47	0.30	90.6
4	7.73	0.28	94.2
6	7.86	0.27	93.8
7	7.66	0.30	93.3
10	7.48	0.31	90.4
Not precipitated by NaCl	0.19	1.67	2.3
Alkali-Soluble Protein			
Precipitated by ethanol			
Extract 2	0.42	2.33	5.1
4	0.93	0.91	11.3
5	1.51	1.08	17.7
6	1.58	1.54	19.3
Residue after extraction	7.62	0.31	92.9

* Calculated from the hydroxyproline content assuming a value of 8.22 for hydroxyproline—N expressed as a per cent of total collagen-N

further this, and other fractions extracted from calf skin by dilute acid and alkaline solutions.

EXTRACTION OF CALF SKIN WITH DILUTE ACID AND ALKALINE SOLUTIONS

Extraction Procedure

A fresh skin from a bull calf about 5 weeks old was used for this investigation. In order to reduce complications due to contamination with keratinous material, elastin, muscle fibres, sebaceous glands and other cellular material, only the middle layer of the skin was used. This was obtained by sectioning 1-inch punches on a freezing microtome. The first few sections from the flesh side were discarded and the following sections collected until the base of the hair follicles was reached. The sections were transferred immediately to 0.1 M disodium hydrogen phosphate solution, pH 9.1 and stored ... punches were sectioned and the wet

... is ... ir ... d

... less than 0.02 mg. nitrogen per ml. corresponding to less than 0.1 per cent ...

extracted ...

buffer, p...

to a low ...

ten extractions with 0.05 M acetic ... finally neutralized, and hydrochloric acid. The macerate was extracted seven times with sodium hydroxide solution, pH 12.0 to 12.2. All extractions were carried out at 0° to 4° C, and were, in ... soluble collagen was

...

a final concentration of 5 per cent. The ... extracts, with the exception of the first, were combined, to remove salts and concentrated. The concentration was in the same cellulose tubing used for dialysis by blowing of air over these at room temperature. The acetic acid ... similarly. The alkaline extracts were neutral- ... ethanol

with phosphate buffer, 20 per cent with citrate buffer, another 8.5 per cent with dilute acetic acid, and 2.2 per cent with dilute alkali, leaving a residue of about 60 per cent. In the citrate buffer and acetic acid extracts nearly all the protein was precipitated by sodium chlor- [illegible]

about half the nitrogen was precipitated by alcohol.

Amino Acid Composition of Protein Fractions

The hydroxyproline and tyrosine contents of some of the fractions were determined in order to gain information as to their identity (see Table III). The method of Neuman and Logan (1950) was used

TABLE III

HYDROXYPROLINE AND TYROSINE CONTENT OF SKIN FRACTIONS

	Hydroxyproline-N	*Tyrosine-N*	*Collagen-N**
	Amino or Imino-acid-N as per cent Total-N		
Citrate-Soluble Proteins			
Precipitated by NaCl			
Extract 1	7 68	0 32	93.4
2	7 95	0 19	97 0
4	7 88	0 29	95 8
7	8 15	0 26	99 3
Not precipitated by NaCl	0 1	0.56	1 2
Acetic Acid-Soluble Proteins			
Precipitated by NaCl			
Extract 2	7 47	0 30	90 6
4	7 73	0 28	94 2
6	7 86	0 27	93 8
7	7 66	0 30	93 3
10	7 48	0 31	90 4
Not precipitated by NaCl	0 19	1 67	2 3
Alkali-Soluble Protein			
Precipitated by ethanol			
Extract 2	0 42	2 33	5 1
4	0 93	0 91	11 3
5	1 51	1 08	17 7
6	1 58	1 54	19 3
Residue after extraction	7 62	0 31	92 9

* Calculated from the hydroxyproline content assuming a value of 8 22 for hydroxyproline-N expressed as a per cent of total collagen-N

further this, and other fractions extracted from calf skin by dilute acid and alkaline solutions.

EXTRACTION OF CALF SKIN WITH DILUTE ACID AND ALKALINE SOLUTIONS

Extraction Procedure

A fresh skin from a bull calf about 5 weeks old was used for this [illegible]

used. This was obtained by sectioning 1-inch punches on a freezing microtome. The first few sections from the flesh side were discarded and the following sections collected until the base of the hair follicles was reached The sections were transferred immediately to 0.1 M disodium hydrogen phosphate solution, pH 9.1 and stored below 4° C. In all about 470 punches were sectioned and the wet [illegible]

less than 0.02 mg. nitrogen per ml. corresponding to less than 0.1 per cent of the total-nitrogen of the skin. The macerate was then extracted seven times with four times its weight of 0 1 M citrate buffer, pH 3.7, until the nitrogen content of the extracts again fell to a low value (0.07 mg. N/ml.) The citrate buffer was followed by ten extractions with *0 05 M acetic acid adjusted to pH 2.8* with hydrochloric acid. The macerate was finally neutralized, and extracted seven times with sodium hydroxide solution, pH 12.0 to 12.3. All extractions were carried out at 0° to 4° C, and were, *in* general, each of 24 hours' duration. The acid-soluble collagen was precipitated from the extracts by the addition of sodium chloride to a final concentration of 5 per cent. The filtrates from the citrate-buffer extracts, with the exception of the first, were combined, dialysed to remove salts and concentrated. The concentration was carried out in the same cellulose tubing used for dialysis by blowing a stream of air over these at room temperature. The acetic acid solutions were treated similarly. The alkaline extracts were neutralized, concentrated and precipitated with three volumes of ethanol.

About 8.5 per cent of the total-nitrogen of the skin was extracted

with phosphate buffer, 20 per cent with citrate buffer, another 8.5 per cent with dilute acetic acid, and 2.2 per cent with dilute alkali, leaving a residue of about 60 per cent. In the citrate buffer and acetic acid extracts nearly all the protein was precipitated by sodium chloride leaving only very small amounts of nitrogen in solution, representing about 0.3 and 0.2 per cent of the total-nitrogen respectively. Of this material remaining in solution only about one-quarter was recovered after dialysis and concentration. In the alkaline extracts about half the nitrogen was precipitated by alcohol.

Amino Acid Composition of Protein Fractions

The hydroxyproline and tyrosine contents of some of the fractions were determined in order to gain information as to their identity (see Table III). The method of Neuman and Logan (1950) was used

TABLE III

HYDROXYPROLINE AND TYROSINE CONTENT OF SKIN FRACTIONS

	Hydroxyproline-N	*Tyrosine-N*	*Collagen-N**
	Amino or Imino-acid-N as per cent Total-N		
Citrate-Soluble Proteins			
Precipitated by NaCl			
Extract 1	7 68	0 32	93 4
2	7 95	0 19	97 0
4	7 88	0 29	95 8
7	8 15	0 26	99 3
Not precipitated by NaCl	0 1	0 56	1 2
Acetic Acid-Soluble Proteins			
Precipitated by NaCl			
Extract 2	7 47	0 30	90 6
4	7 73	0 28	94 2
6	7 86	0 27	93 8
7	7 66	0 30	93 3
10	7 48	0 31	90 4
Not precipitated by NaCl	0 19	1 67	2 3
Alkali-Soluble Protein			
Precipitated by ethanol			
Extract 2	0 42	2 33	5 1
4	0 93	0 91	11 3
5	1 51	1 08	17 7
6	1 58	1 54	19 3
Residue after extraction	7 62	0 31	92 9

* Calculated from the hydroxyproline content assuming a value of 8 22 for hydroxyproline—N expressed as a per cent of total collagen-N

for the determination of hydroxyproline, and that of Udenfriend and Cooper (1952) for tyrosine. The proteins precipitated by the addition of sodium chloride to the acid extracts all had a high

sine contents suggesting that they mainly consisted of some non-collagenous protein.

An approximate value for the collagen content of the various

content of the fractions was increased. In the alkali-soluble fractions the collagen content increases as the extraction proceeds suggesting that a non-collagenous type protein is preferentially extracted in the early stages.

The hydroxyproline content of the extracted skin residue is still lower than that of the purified citrate-soluble collagen and the tyrosine content is higher, suggesting that about 7 per cent of non-collagenous protein may still be present in this material.

The amino-acid composition of the three non-collagenous protein fractions was determined by the method of Moore and Stein (see

and arginine contents are much lower, and the lysine, histidine, aspartic acid, leucine, isoleucine and valine contents are much

TABLE IV

THE AMINO-ACID COMPOSITION OF PROTEIN FRACTIONS EXTRACTED FROM CALF SKIN

	Citrate-Soluble Protein Precipitated*	*Citrate-Soluble Protein* Not precipitated	*Acetic Acid Soluble Protein not precipitated*	*Alkali-Soluble protein*
	(Amino Acid-N as per cent Total-N)			
Glycine	27.48	6.18	4.44	6.39
Alanine	8.84	8.46	5.70	5.13
Leucine	1.93	5.03	4.49	7.31
Isoleucine	0.84	2.98	1.96	3.81
Valine	1.53	4.30	3.21	4.76
Phenylalanine	0.95	2.18	1.88	2.65
Tyrosine	0.22	0.58	1.67	2.33
Tryptophan	not found	not determined		
Serine	3.19	4.01	3.76	4.77
Threonine	1.47	3.99	3.63	3.73
Cystine		not found		
Methionine	0.41	0.23	0.23	0.57
Proline	8.95	2.86	not found	4.17
Hydroxyproline†	8.22	0.10	0.19	0.42
Arginine	15.16	7.67	5.20	14.16
Histidine	0.45	2.56	5.64	3.62
Hydroxylysine	1.26	?	?	?
Lysine	3.87	7.07	5.03	7.75
Aspartic acid	3.60	7.28	7.14	8.48
Glutamic Acid	5.93	6.77	0.37	8.03
Ammonia (M and S)	(2.74)	12.78	9.56	(7.66)
Amide†	2.92	—	—	8.20
Glucosamine (M and S)	—	(0.18)	(7.27)	(0.63)
Galactosamine (M and S)	—	—	—	(1.02)
Hexosamine (Elson and Morgan)	0.01	1.33	10.14	2.90
Unknown‡	—	0.04	0.23	0.53
Totals (values in brackets not included)	97.23	86.40	74.47	99.69

* Taken from Bowes, Elliott and Moss, *Biochem J*, 1955, 61, 143
† Direct determinations
‡ Calculated on the basis of 1 nitrogen atom per mole.

[illegible]

serine and threonine. With the acetic acid-soluble fraction it is likely that some ammonia will have arisen from destruction of

hexosamine. The absence of any detectable proline and the very low value for glutamic acid in this fraction is also surprising. Low values for these acids were also found in the corresponding fraction of tendon examined in the preliminary experiment.

The high value for glucosamine in the acetic acid-soluble fraction was confirmed by independent determination of hexosamine by the Elson and Morgan method on a sample of the same hydrolysate. After mild hydrolysis with 2 N hydrochloric acid a rather higher value was obtained (Table V).

TABLE V

HEXOSE AND HEXOSAMINE IN CALF SKIN FRACTIONS

	gm per 100 gm Nitrogen			*gm per 100 gm Protein*	
	Hexose	*Glucosamine*	*Galactosamine*	*Hexose*	*Hexosamine*
Fresh Calf Skin	0·2	0·4	0·3	0·03	0·1
Protein extracted at pH 3·7					
Precipitated	0·1	0·1		0·02	0·01
Not Precipitated	3·2	11·7	5·4	0·5	2·7
Protein extracted at pH 2·8					
Not precipitated	18·6	129·8 (probably nearly all glucosamine)		3·3	23·2
Protein extracted by alkali and precipitated by ethanol	0·3	14·1	22·9	0·05	6·1
Extracted Calf Skin Residue	0·03	0·2	0·1	0·01	0·06

The amino-acid composition of the alkali-soluble fraction was also obviously different from that of collagen, though the presence of some hydroxyproline suggests that it contains a small amount of collagen, about 5 per cent, in the case of the particular sample examined. Even when allowance is made for the presence of this ... tyrosine proline, glutamic ... he acid-soluble ... material were ...

to the two peaks.

The hexosamine and hexose contents of the various fractions were determined independently after milder conditions of hydrolysis with

2 N hydrochloric acid for 16 hours at 105° C. The hexosamines and hexoses were separated from the amino acids on an ion exchange resin (Dowex 50) and determined by modifications of the Elson and [illegible]

mine nitrogen.

[illegible]

that appreciable amounts of hexoses and hexosamines may have been lost by diffusion through the cellulose membranes during dialysis and concentration. Except in the alkali-soluble fraction, the glucosamine content was greater than the galactosamine content. Hexuronic acids were not detected in the hydrolysates of any of these fractions, but as these substances are readily destroyed by acid this is no proof of their absence. If hexuronic acids equivalent to the hexosamine were present in the acetic acid-soluble fraction, however, they would almost certainly have been detected. Unfortunately there was insufficient material available to make a direct determination of uronic acids.

Extraction with Dilute Alkali

Some further experiments were carried out on the extraction of alkali-soluble protein. In these the whole thickness of the calf skin was used. The skin was closely shaved to reduce complications due to the solution of keratinous material, cut into pieces about 1 cm. in area and given a preliminary extraction with six changes of 0.1 M phosphate buffer, pH 9.0. Samples of the skin were then extracted with sodium hydroxide or calcium hydroxide solution at pH 12.5 at three different temperatures, 4°, 20° and 30° C. The total-nitrogen, ammonia-nitrogen, hydroxyproline-nitrogen, and tyrosine-nitrogen of the solutions were determined (see Table VI).

As before, the hydroxyproline values indicate that it is mainly a non-collagenous protein which is extracted. The amount of collagen dissolved increases with temperature to a greater extent than does

hexosmine. The absence of any detectable proline and the very low value for glutamic acid in this fraction is also surprising. Low values for these acids were also found in the corresponding fraction of tendon examined in the preliminary experiment.

The high value for glucosamine in the acetic acid-soluble fraction was confirmed by independent determination of hexosamine by the Elson and Morgan method on a sample of the same hydrolysate. After mild hydrolysis with 2 N hydrochloric acid a rather higher value was obtained (Table V).

TABLE V

HEXOSE AND HEXOSAMINE IN CALF SKIN FRACTIONS

	gm per 100 gm Nitrogen			*gm per 100 gm Protein*	
	Hexose	*Glucosamine*	*Galactosamine*	*Hexose*	*Hexosamine*
Fresh Calf Skin	0 2	0 4	0 3	0 03	0 1
Protein extracted at pH 3 7					
Precipitated	0 1	0 1		0 02	0 01
Not Precipitated	3 2	11 7	5 4	0 5	2 7
Protein extracted at pH 2 8					
Not precipitated	18 6	129 8 (probably nearly all glucosamine)		3 3	23 2
Protein extracted by alkali and precipitated by ethanol	0 3	14 1	22 9	0 05	6 1
Extracted Calf Skin Residue	0 03	0 2	0 1	0 01	0 06

The amino-acid composition of the alkali-soluble fraction was also obviously different from that of collagen, though the presence of some hydroxyproline suggests that it contains a small amount of collagen, about 5 per cent, in the case of the particular sample examined. Even when allowance is made for the presence of this

to the two peaks.

The hexosamine and hexose contents of the various fractions were determined independently after milder conditions of hydrolysis with

2 N hydrochloric acid for 16 hours at 105° C. The hexosamines and hexoses were separated from the amino acids on an ion exchange resin (Dowex 50) and determined by modifications of the Elson and Morgan and Anthrone methods respectively (see Moss, 1955).

[illegible]

mine nitrogen.

Both the hexose and hexosamine contents of the original skin, the acid-soluble collagen and the final extracted residue are low, and [illegible]

sis and concentration. Except in the alkali-soluble fraction, the glucosamine content was greater than the galactosamine content. Hexuronic acids were not detected in the hydrolysates of any of these fractions, but as these substances are readily destroyed by acid this is no proof of their absence. If hexuronic acids equivalent to the hexosamine were present in the acetic acid-soluble fraction, however, they would almost certainly have been detected. Unfortunately there was insufficient material available to make a direct determination of uronic acids.

Extraction with Dilute Alkali

Some further experiments were carried out on the extraction of alkali-soluble protein. In these the whole thickness of the calf skin was used. The skin was closely shaved to reduce complications due to the solution of keratinous material, cut into pieces about 1 cm in area and given a preliminary extraction with six changes of 0.1 M phosphate buffer, pH 9.0. Samples of the skin were then extracted with sodium hydroxide or calcium hydroxide solution at pH 12.5 at three different temperatures, 4°, 20° and 30° C The total-nitrogen [illegible]

Dr. Bowes thought that there were also differences in tyrosine and leucine, that is, in those amino acids probably present in relatively high concentrations in the non-collagenous component.

Referring again to the unreliability of methods for determining sugars in connective tissue, Dr. Meyer said that the orcein method might give different values to the anthrone method for four reasons·

(1) the method depends on the standard used;
(2) it depends on the amount of browning;
(3) it depends on the type of sugar;
(4) it depends on the type of linkage.

He quoted the apparent difference in hexose content of heparin and disulphated heparin as determined by the orcein method The results for [illegible] the uronic acid.

Dr Bowes confirmed Dr. Meyer's suspicion that the values might be unreliable The amount of hexosamine estimated to be present in the acetic acid-soluble fraction was probably greater than the amount of hexosamine present in the original skin sample indicating that one at least [illegible] me [illegible] ild [illegible] not in fact observed.

Dr. Bowes pointed out that there was some loss of amino acids during the dialysis and that one should not, therefore, attempt to make too detailed a comparison of the amino-acid compositions of the different fractions and draw conclusions from this.

Dr. Orekhovitch thought that the differences between the fractions might be due to the presence of peptides rich in these amino acids (tyrosine and histidine).

Dr. Bowes said that small peptides would probably have been removed during dialysis, but it was possible that these amino acids might be present in large peptides which had been split off from the collagen. If such were the case the hydroxyproline must be concentrated in the remaining part of the chain, and although Dr. Grassmann's work indicated [illegible] of hydroxyproline residues are located near one another, [illegible] composition required

Dr Orekhovitch said the peptides might be weakly bound to proteins and, therefore, not separated by dialysis. For example, serum albumin

adsorbs amino acids strongly, and they can only be separated by repeated fractional recrystallization

Dr. Bowes said that the fractions were probably not homogeneous, and apart from saying that one or possibly two relatively large peptides or protein fractions of a non-collagenous nature had been isolated from skin, she did not think at this stage it was justifiable to draw further conclusions from the analysis of these fractions.

Dr. Grassmann said that if one avoided the use of concentrated strong acids in hydrolysis, the amino acids in the hydrolysate could be separated as their DNP derivatives from hexoses and DNP amino sugars on an ion exchange column

Dr. Meyer thought that in the materials with which they were concerned, even hydrolysis with 1 N acid at 100° for 8 hours (Dr. Grassmann's hydrolysis conditions), some destruction of sugars, particularly uronic acids, might occur

Dr. Grassmann replied that, except for uronic acids which he had not been concerned with he had found these conditions reliable. Even after hydrolysis for 20 hours, the amount of hexose estimated did not fall

Dr. Gross thought that a qualitative examination of the hydrolysate should be done first so that one could choose appropriate standards fo subsequent quantitative measurements. Using synthetic mixtures carbohydrate and amino acids, he had found a gradual loss of hexoses as the mixture was hydrolysed with 2 N acid for increasing times and suggested that it might be due to impurity of the hydrochloric acid he had used He thought that, for comparative purposes, more reliable results could be obtained by measuring the orcein colour directly by Friedman's method

Dr Neuberger pointed out that more ammonia was obtained in the Moore and Stein analysis of the acetic acid-soluble collagen than one would expect from the sum of the aspartic and glutamic acid content He suggested that ammonia might arise from some source other than amide groups and asked if Dr. Bowes had determined amide nitrogen by the standard method of hydrolysis with 2 N acid for three hours

Dr Bowes said that some ammonia might have come from decomposition of hexosamine but that no direct amide nitrogen determinations had been done owing to shortage of material.

Dr Consden said he had obtained results similar to those of Dr. Bowes by extracting washed human subcutaneous tissue with alkali. The protein extracted was largely non-collagen since it contained tryptophan, and was relatively rich in tyrosine and poor in hydroxyproline The extract also contained 10-20 per cent carbohydrate, much of which was a mixture of sulphated acid polysaccharides. These were predominantly non-uronic acid containing and were comprised of hexosamine and hexose

(galactose or galactose+mannose). Similar protei
mixtures were liberated after dig
With dilute potassiun
polysaccharide and non- ... extrac
The remaining 50 per cent could not be extracted
months. It therefore appeared that in loose connective
fibres were closely associated with two types of non-co
acid polysaccharide, one of which was much more tigh
other.

In reply to Dr. Consden, Dr. Bowes said she had no ev
collagen contained no tyrosine.

Dr. Grassmann said he had isolated a peptide from
contained 42 amino acids. Each chain contained 2 tyrosi
amount of peptide he had isolated was about 1.5 per cent
and it might be present to the extent of 3 per cent.
there must be about 0.15 per cent tyrosine in collagen (
acid sequence) Thi

Dr. D. S. Jackson
about the same am
was only a small a
actually part of the collagen molecule. thought that

Dr Robb-Smith and Dr
chemical techniques migh
tissues as they passed throu

Dr. Bowes said that preliminary work on these lines had ... procedu
Staining with haem-alum indicated that the fibroblasts were
intact up to the end of acetic acid extraction, but disappeared
alkaline extraction.

PROCOLLAGENS AS BIOLOGICAL PRECURSORS OF COLLAGEN AND THE PHYSICO-CHEMICAL NATURE OF THESE PROTEINS

V. N. OREKHOVITCH AND V. O. SHPIKITER

In our previously published work we made a hypothesis on the transformation of procollagen into collagen. A careful study of similar data published during recent years by other laboratories and their comparison with our data tends to confirm our original ideas concerning the biological role of the procollagens.

In this paper we shall give the results of our research concerning procollagens and, in the first place, their properties and their modes of transformation.

We shall begin by giving the results of the studies concerning the physico-chemical nature of procollagens and also all data concerning the molecular weight and the dimensions of the particles of these proteins. For some time it was considered that the molecular weight of procollagen isolated by the method used in our laboratory (extraction by an acid-buffer solution of citrate followed by dialysis against tap water) is 70,000 and the length of the molecules 380 Å (Bresler, Finogenov and Frenkel, 1950). Later, however, it was seen that these values corresponded not to native procollagen but to its thermal denaturation products, as the procollagen solutions were prepared at about 40° C. We now know that during such treatment the procollagen particles break down. In connection with this we started in 1954 various researches on the molecular weight of native procollagen (Orekhovitch and Shpikiter, 1955).

With this in view, we studied sedimentation, diffusion and viscosity of procollagen in citrate buffer solutions with a pH of 3.6 containing 1 per cent of calcium chloride or 0.5 M urea. The coefficients of sedimentation were measured for a series of concentrations. By extrapolation to infinite dilution we found that $s = 3.05$-3.25 Svedberg units. For the diffusion constant we have obtained $D = 0.35$-$0.40 \; 10^{-7}$ cm.^{2}/sec., and one of these determinations was made with the Tsetkov polarizing interferometer (Tzvetkov, 1951) with a concentration of protein of 0.02 per cent, that is to say a concentra-

tion which allows one to consider the particles in solution as kinetically *independent. Viscosity* was measured by the U-shaped capillary viscosimeter with different shear gradients (from 2500 to 100 $seconds^{-1}$); our data enabled us to calculate the value of intrinsic viscosity $[\eta] = \eta_{sp}/c$, with $c \to 0$ (η_{sp}-extrapolated to $q = 0$ and with c-g/100 *ml.) which is found to equal 16–17*. The molecular weight calculated from these data corresponded to 700,000 and the length of the molecule to nearly 7000 Å. The difficulties we met in our research on procollagen *solutions by physico-chemical methods do not allow us to consider these data as exact. It is more accurate* to speak of an order of magnitude. It should be said that similar results were obtained by the Japanese worker Noda (1955) for acid soluble collagen similar to procollagen. This worker obtained $s = 3.5 \cdot 10^{-13}$; $D = 0.5 \cdot 10^{-7}$ *cm.²/sec. and calculated* $M = 710{,}000$ and length of the particles nearly 5000 Å. For ichthyocol (very similar to procollagen) extracted by a citrate buffer solution, Gallop (1955) obtained $s = 2.85 \cdot 10^{-13}$, $[\eta] = 13.2$, the molecular weight determined by light scattering $1 \cdot 7 \cdot 10^{6}$, and the length of particles = 4000 Å.

Boedtker and Doty (1955) have also studied the citrate extract of ichthyocol and obtained $s \approx 2.85 \cdot 10^{-13}$, $[\eta] = 11.5$. On the basis of these data, and assuming a hydration of 34 per cent and a high asymmetry, these workers obtained a molecular weight of 300,000, remarkably similar to the results obtained by the study of osmotic pressure (300,000) and light scattering (340,000). The length of the molecule was found to be 3000 Å by flow-birefringence. The similarity of the average molecular weights obtained from osmotic pressure and light scattering measurements indicates that this protein is very homogeneous. In addition, several papers were published on the determination of the molecular weight of procollagen proteins by the method of light scattering, and *values of* the order of 10^{6}–10^{7} were obtained. However, one should accept with great caution values obtained by the light scattering method because of the great difficulty of freeing the solution from dust, the presence *of which alters the results considerably*. For the time being we shall not analyse these data for the determination of molecular [illegible] to stress that the molecular weight and the

[illegible] proteins, the constants of sedimentation $s \approx 3 \cdot 10^{-13}$ and values of intrinsic visco-

sity $[\eta] = 15$ are very characteristic, and it is for this reason that these data may be used for the identification of procollagen.

To conclude this part, concerning the physico-chemical nature of procollagen, we shall quote a few of our results relating to its macro-structure. When solutions of procollagen are heated at a certain temperature (according to the pH of the milieu) one observes a considerable lowering of viscosity (at pH = 4 the temperature is around 37° C.; at pH 2 the temperature is around 30–32° C.; at pH 6 around 40° C.); urea in a concentration of 3 M at pH 4 lowers he temperature at which viscosity drops to 33° C. and at a concen-:ration of 6 M the temperature at which viscosity drops falls to 23° C. The same lowering of viscosity was seen with concentrated solutions of salts (for example with 5 M KCNS, KI, $CaCl_2$, etc.). The same action occurs with hydrogen peroxide at a concentration of approximately 20 per cent. In all these cases the drop in the viscosity is accompanied by irreversible modifications of the procollagen molecules, viz. breakdown into simpler components.

The products of disintegration differ considerably from the native procollagen. Contrary to it, these products give thixotropic gels, are not precipitated by 30 per cent acetone or by 4 per cent sodium chloride, and by their properties are related to gelatin.

During ultracentrifugation, after heating the solution of procolla-gen (citrate-buffer solution at pH 4 containing 3 M urea at 30° C.) for 10 minutes, two components are detected with sedimenta-tion constants $s = 2.4 \cdot 10^{-13}$ and $s = 3.5 \cdot 10^{-13}$. We obtained similar results by heating a suspension of procollagen in a phosphate buffer solution at pH 8 at 70° C. during 10 minutes with subsequent addi-tion of a solution of 2 M of KCNS to a final concentration of 1 M (see Fig. 1*a*) or by dissolving the procollagen at room temperature in a buffer solution containing 5 M of KCNS with subsequent addition of buffer until a concentration of 1 M of KCNS was reached (see Fig. 1*b*). The heating of the suspension of procollagen and the action of KCNS (by adding to the heated solution dry KCNS to a concentration of 5 M with subsequent dilution of the solution to 1 M KCNS) gives a similar picture, as can be seen from Fig. 1*c*, the procollagen breaks down into two components. These results lead us to think that the compounds detected by ultracentri-fugation are constituent elements of the molecules of native pro-collagen. It is interesting to note that the character of the disintegra-tion products of the molecule of procollagen is similar and does not

depend on the nature of the substances which act upon it. This is to say that the same results are obtained by the action of heat, of urea, or of high concentrations of salts. At the present time, we are continuing these studies, and we intend to isolate these components.

We shall now consider the data which confirm our hypothesis that procollagen is a biological precursor of collagen.

First, in favour of this hypothesis, we have the data relating to

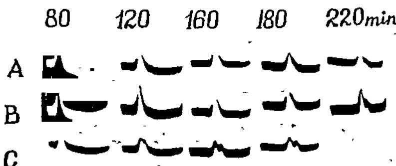


Fig 1

Sedimentational diagrams of procollagen breakdown products Svedberg ultracentrifuge, 56,000 r p m, rotor temperature 28-30° C Solvent 1/15th M phosphate buffer, pH 8 + 1 M KCNS

A, heated at 70° C for 10 min.

B, dissolved in 5 M KCNS

C, heated at 70° C for 10 min, then treated with 5 M KCNS

Second, in certain pathological states, for example in cases of

the synthesis of procollagen, we have studied in our laboratory the quantity of this protein in guinea-pigs suffering from scurvy. We have established that in healthy animals the skin contains 10 per cent

...eight of desiccated and defatted

the amount is 5 per cent. The

both groups of animals remains

constant when compared to the quantity of collagen in the skin of healthy animals at the time of the onset of scurvy. More convincing data on the inhibition of the synthesis of procollagen in the skin of animals suffering from scurvy were obtained in experiments with radioisotopes. We injected under the skin of healthy and affected guinea-pigs glycine containing radioactive carbon on the carboxyl group. After a variable lapse of time following the injection of labelled glycine the animals were sacrificed and the procollagen and collagen (gelatin) isolated from their skin. The radioactivity of these proteins was determined In Table I we give the results and it can

TABLE I

— OF INCORPORATION OF ^{14}C GLYCINE IN THE PROCOLLAGEN AND COLLAGEN OF HEALTHY AND SCORBUTIC GUINEA-PIGS (IMP./MIN. FOR 10 MG. OF PROTEIN)

Substance	*Time elapsed after injection of the amino acid (hours)*							
	3	6	10	18	24	120	168	384
Procollagen								
Scorbutic animals	3	6	4	2	1	0	3	0
Healthy animals	45	134	140	192	190	122	89	70
Collagen								
Scorbutic animals	1	1	0	0	2	—	3	3
Healthy animals	9	12	13	12	16	—	23	25

be seen that ^{14}C glycine is not found in the procollagen of animals suffering from scurvy, that is to say that the synthesis of this protein is completely inhibited, which corresponds to an arrest in the formation of collagen.

Third, the study of the rate of incorporation of radioactive glycine in the procollagen and collagen of healthy animals following at various intervals after the injection of this amino acid confirms our concept that procollagen is a precursor of collagen. As can be seen from Table II, the greatest radioactivity of procollagen is found after 12-24 hours after the injection of radioactive glycine in the animals.

In the following days, radioactivity of procollagen diminishes rapidly, and on the fortieth day it reaches values lower than those for collagen which are found 3 hours after the injection of radioactive glycine. For collagen it is the opposite process which is characteristic.

The longer the time following injection of radioactive glycine, the

Table II

RATE OF INCORPORATION OF ^{14}C GLYCINE IN THE PROCOLLAGEN AND COLLAGEN OF GUINEA-PIGS (IMP./MIN. FOR 10 MG. OF PROTEIN) AT VARIOUS TIMES AFTER THE INJECTION OF AMINO ACID

Observed object	*Time elapsed after injection of amino acid (in hours)*											
	3	6	12	24	96	480	840	840	960	960	960	960
Procollagen												
1st extraction	71	119	130	157	97	84	32	18	7	8	6	14
2nd ,,	62	114	110	119	75	—	44	37	39	20	12	38
3rd ,,	40	79	84	84	52	52	40	41	41	44	27	40
4th ,,	50	60	64	64	42	44	52	43	71	38	33	41
5th ,,	35	65	60	64	53	—	55	35	70	—	35	33
6th ,,	27	32	38	48	32	38	32	31	54	29	30	35
Collagen	12	17	22	26	30	40	43	39	80	53	44	44

higher the radioactivity of the collagen isolated from these animals. After 3 hours, the radioactivity of collagen corresponds to 12 imp./min for 10 mg. of protein; 40 days after, it corresponds to 80 imp./min., that is nearly seven times more. If we compare these data with the radioactivity of the first extraction of procollagen and collagen, one is struck by the following fact: 24 hours after injection in the organism of radioactive glycine, the radioactivity of procollagen begins to decrease, being practically nil around the fortieth day, whereas for collagen it is towards the fortieth day that it is maximum. At that time, the radioactivity of collagen is approximately twelve times greater than that of procollagen. If we take into account the fact that the incorporation of radioactive glycine in these proteins takes place only at the time of their formation, this [illegible] the following fashion. In the first [illegible] is of pro- [illegible] d it is for

that reason that the protein fraction obtained from the first extraction contains a radioactive procollagen. On the second, third and other extractions, it is principally the protein substances synthesized [illegible]

observed in reality. As procollagen [illegible] passing through intermediary protein substances, the radioactivity of these substances and of collagen increases. After a certain time the procollagen synthesized at the time of the greatest concentration of

radioactive glycine in the organism will transform itself into collagen which will enrich itself in radioactive carbon. At that time the

seen in our experiments.

gen, tropocollagen, etc.). Sometimes these are taken for real precursors of collagen whereas the citrate-soluble collagen (procollagen according to our terminology) is sometimes taken for collagen. We believe it is important to discuss the relationship of these proteins and to clarify as far as possible the question of the precursors of collagen

At the time we published our results concerning the study of the metabolic activity in procollagens with the use of ^{14}C glycine, Professor Neuberger was publishing results similar to our own. Subsequently, Professor Neuberger and his co-workers (Harkness *et al.*, 1954) expressed the opinion that other precursors of collagen may exist. Among other points, he relates to these other predecessors the alkaline-soluble fractions of collagen Gross, Highberger and Schmitt (1954), on the basis of electron microscopic research on various structural forms of collagen precipitated under various conditions from tissue extracts, made the hypothesis that primary structural units having a length of 1500-3000 Å are present

extracted by neutral saline solutions. It was supposed that this tropocollagen passing into solution exists in the ground substance of connective tissue whereas the 'procollagen of Orekhovitch' comes from newly formed collagen fibrils (Gross *et al*, 1955).

of these fibres is greatly altered by extraction with an acid buffer solution.

The following scheme, suggested by Grassmann (1955), thus ensues:

Tropocollagen – Procollagen – Collagen.

We present this scheme, for greater clarity, in tabular form:

TABLE III

Suggested names	*Characteristics*
Tropocollagen	[illegible] stance of connective tissue
Procollagen	Extracted by acid solutions and by acid buffer solutions. Precipitated by neutral and alkaline solutions. Gives a periodic cross striation of 650 Å. Deaggregates when heated to 37° C in acid solutions. Metabolically less active. Present in newly formed collagen fibres
Collagen	'Condensed' fibres. Insoluble in solutions weakly alkaline or neutral. Gives cross periodic striation of 650 Å. Very little metabolic activity. Constitutes the principal mass of collagen fibres

In the discussion of this scheme, it is essential to quote the data which characterize the physico-chemical nature of tropocollagen and compare them with those of procollagen. We have isolated by [illegible] and have com- [illegible] thod. We have [illegible] stals' identical to [illegible]

soluble collagen in 0.1 M citrate buffer solution at pH 4 containing 1 per cent of $CaCl_2$ we obtained $s = 3.1 \cdot 10^{-13}$ and $[\eta] = 13.0$ (for procollagen in these conditions $s = 3.05 \cdot 10^{-13}$ and $[\eta] = 17.5$). We may conclude that these two proteins, by their physico-chemical properties, are practically identical.

We may add that Jackson and Fessler (1955) found for collagen soluble in saline solution with a neutral pH a value of $s = 3.23 \times 10^{-13}$, a molecular weight of the order of 10^6 and a length of particles by birefringence corresponding to 4000 Å. If we take into account

the errors of the methods and the differing conditions of these experiments we may say that the physico-chemical characteristics of tropocollagen and of procollagen correspond very closely and, for this reason, the particles of these proteins do not differ fundamentally from each other and have the same physico-chemical nature. [illegible]

citrate (at pH 5) in considerable quantity and that, if an alkaline solution was added until pH 7 was *reached, the solution was stable.* However, when it is heated up to 37° C., a precipitate of protein is [illegible]

fundamentally different from that of procollagen.

The periodicity of cross striation is not a good criterion of the nature of these primary particles. This is particularly clear in the experiments of Schmitt and others (1953) on the reciprocal transformation of various structural forms of collagens precipitated under different conditions.

With regard to the metabolic activity of tropocollagen we must point out that in extracting finely minced skin tissue with a citrate buffer solution (without prior treatment with phosphate) we have obtained in the first extract a tropocollagen with a greater metabolic activity.

GROUP DISCUSSION

[illegible] gested that Dr Orekhovitch's extraction procedures produced both neutral-extracted collagen and citrate-extracted collagen and that, although they were both substantially referring to the same product, theirs was the more representative precursor since the properties of their particular preparation could be accounted for on the assumption that it was the true precursor of collagen fibres.

Dr Orekhovitch explained that in his method the citrate extraction was preceded by extraction with phosphate or other neutral solution He felt that such preliminary extraction would remove all extraneous

material leaving the procollagen to be extracted by the subsequent citrate solution.

DR D. S. JACKSON asked how Dr. Orekhovitch explained the fact that in the neutral extract there was a collagen component which took up radioactive ^{14}C glycine at a greater rate than his citrate extract.

DR OREKHOVITCH stated that the high activity of the fraction extracted by neutral solution was due to the presence of the serum proteins alone

DR. OREKHOVITCH stated that he had seen no numerical data on this matter, whereupon DR. NEUBERGER quoted experiments from which it could be shown that the specific activity of radioactive glycine isolated

in the serum protein. If Dr Orekhovitch's observations were cor

one would expect a hundred times greater concentration in the serum proteins than in the alkali-soluble collagen and this was not observed. The only explanation is the existence of a protein with an exceptionally high specific activity

DR. GROSS drew attention to the fact that Dr. Orekhovitch had stated that the neutral salt extract contained little collagenous material. He pointed out that, if extracts were made from the tissues of rapidly growing animals, using a buffer tissue ratio of 2 : 1, the extract contained as much as

ation of growth

his animals. On the question of terminology, he felt that Dr. Orekho-vitch's use of the word 'tropocollagen' needed clarification The term tropocollagen was not intended to imply precursor or source of a collagen unit but was to be taken as referring to a building block. Tropocollagen

has never been incor-

representing material

id-extracted collagen,

e from salt-extracted

collagen since electron-microscopical examination of the fibres after

neutral salt-soluble collagen is the actual precursor of the

DR. OREKHOVITCH answered, firstly, that all his animals were in a good

nutritional state, hence starvation could not be taken as the reason for his failure to find procollagen in alkaline extracts. The morphological characteristics of the fibrils should not be confused with their chemical analysis, and hence procollagen should be regarded as a chemical rather than a structural precursor. Dr. Orekhovitch then asked if tropocollagen could be classed as a micromolecule, to which Dr Gross replied no, it was 2000-3000 Å long by 15 Å wide and represents at the present time the smallest collagen unit that can be converted directly into a fibril: any smaller particle being probably a breakdown product which cannot be directly built into a fibril

In reply to a question by Dr. Orekhovitch, Dr. Gross stated that tropocollagen could be compared with the whole of the insulin molecule of molecular weight 34,000, and not to the 6000 molecular weight portion thereof. He added that he was not sure whether globular protein molecules should be directly compared with fibrous protein molecules.

Dr Neuberger said that he regarded tropocollagen as comparable with the insulin monomer from which the Boston workers had shown the fibrous form of insulin to be built up

(monomers). Collagen and feather keratin provide the cases best characterized to date. The monomers of the fibrous proteins are more asymmetric and often more difficult to disperse for physico-chemical characterization than are those of the soluble, crystallizable ones, which

tween this state of affairs and that occurring in the myosin and tropomyosin field. To which Dr Gross replied that there was no evidence that tropomyosin could be converted into myosin, since, as Dr Neuberger added, Bailey has shown that there is no close similarity between

'tropocollagen' to be
more specific in that

Dr. Bear remarked that there is little evidence as yet that the collagen molecules isolated by various extraction procedures are in fact significantly different chemically. For this reason, biochemists may be reluctant to

THE STRUCTURE OF A CHONDROITIN SULPH[...] COMPLEX FROM CARTILAGE

H. M. Muir

Chondroitin sulphate was extracted from hog laryngeal cart[...] with 10 per cent $CaCl_2$ by the method of Blix and Snellman (19[...] and purified by precipitating from dilute solution twice w[...] $Co(NH_3)_6Cl_3$ and twice with 5-aminoacridine at pH 4, the fr[...] polysaccharide being regenerated each time by shaking with catio[...] exchange resins.

The substance appeared to be electrophoretically homogeneou[...] and its aqueous solution had a high viscosity (0.5 per cent soln. η rel. = 6.1 at 25° C.). The analytical figures showed that there was 1.64 per cent of non-hexosamine nitrogen, equivalent to about 10 per cent of amino acids (Table I).

TABLE I

CHONDROITIN SULPHATE FROM HOG LARYNGEAL CARTILAGE

	Found (per cent)	Found after papain treatment	$C_{14}H_{19}O_{14}NSNa_2\,4H_2O$
Hexosamine (Elson and Morgan)	29.18	31.5	31.1
Na (flame)	6.62	7.15	8.01
SO_4 (Benzidine)	14.46	16.55	16.68
N (Kjeldahl)	3.92	2.93	2.44
Non-hexosamine nitrogen by difference	1.64	0.47	—

The viscosity of the aqueous solution was unaffected by heating for 30 minutes at 100° C., but was quickly destroyed by weak alkali at room temperature, while even at pH 8.5 the viscosity began to fall slowly. No flocculation occurred either on heating or treatment with protein precipitants.

Crystalline proteolytic enzymes affected the viscosity to different degrees (Table II).

TABLE II

EFFECT OF ENZYMES ON VISCOSITY OF CHONDROITIN SULPHATE (1 PER CENT SOLUTION)

Enzyme (10 µg/ml)	*Time after adding enzyme (minutes)*	*Per cent fall in viscosity*
Trypsin	100	24
"	200	25 7
Chymotrypsin	160	17
Carboxypeptidase	120	6
Pepsin	100	48
Papain	10	80
"	100	84

[illegible]

Inhibition by sulphydryl reagents showed that the effect was due to papain itself and not to any contaminating papaya lysozyme (Smith, Kimmel, Brown and Thompson, 1955) 4-6 per cent of dialysable

[illegible]

pact spot of $R_f = 0.58$ was obtained, while the undegraded material remained at the origin. There appeared to be a correspondence between loss of viscosity and chromatographic behaviour, as the mobile spot was not produced either by heating or by those enzymes which did not markedly affect the viscosity. Pepsin had an intermediate effect and produced a mobile spot, a spot at the origin and intermediate streaks

Two-way paper chromatography together with paper electrophore[illegible]
12 am[illegible]
and a[illegible]
phenylalanine and a trace of tyrosine was found. No tryptophan could be identified by U.V. absorption of the polysaccharide. The amino acids present in largest quantity were glutamic and aspartic acids, serine, glycine, alanine, valine and leucine. Threonine and proline were present in smaller amounts and a small amount of lysine was identified by paper electrophoresis at pH 8.6 No hydro-

xyproline was detected by the Neuman and Logan procedure and no neutral sugars could be detected after mild acid hydrolysis by paper chromatography.

The papain digested product after prolonged dialysis was twice precipitated from dilute solution with 5-aminoacridine, converted to the sodium salt and finally precipitated with 70 per cent alcohol in the presence of sodium acetate. The analytical figures show a small residue of 2.17 per cent of non hexosamine nitrogen (cf. Table I) [illegible]

exception of serine which now stood out on the chromatogram. The papain digested material was treated overnight with 0.1 N alkali at room temperature, neutralized and dialysed, and the contents of the sac hydrolysed with acid. Paper chromatography of the hydrolysate showed that the residual serine had now been removed.

These results would suggest that the chondroitin sulphate which is relatively easily extracted from cartilage is a complex consisting largely of polysaccharide cemented by a small amount of peptide or protein. This complex is rapidly destroyed by papain leaving a polysaccharide which still appears to retain serine. This serine is easily removed by 0.1 N alkali and since the chondroitin sulphate complex also loses its viscosity in 0.1 N alkali, it is possible that the protein or peptide is attached to the polysaccharide through serine by an alkali labile bond.

GROUP DISCUSSION

[illegible] which were visible on the [illegible] ich Dr. Muir [illegible] bases on the chromatogram. One of the spots concerned was galactosamine and the other was also a sugar, as yet unknown.

Dr. Partridge *stated that by employing ion-exchange resin to remove* the collagen, he had obtained a very similar protein polysaccharide complex from bovine nasal septa The protein complex was stable to relatively high salt concentrations but could be broken by cold alkali or *boiling dilute acetic acid.* The analyses were similar to those of Dr Muir but the protein did contain basic amino acids

In answer to a further question by Dr. Partridge, Dr. Muir gave the

composition of her chromatographic solvent for C S A. as 45 per cent n-propanol, 55 per cent 0.2 M boric acid, and the sugars were stained with toluidine blue

DR. MEYER suggested that the basic spot might be chondrosin Dr. Meyer also inquired whether any electrophoresis figures had been obtained, to which DR MUIR replied that the material ran slightly differently from C.S.A.

DR. PARTRIDGE gave similar results and suggested that part of the polysaccharide was bound and part free and that the two had similar electrophoretic mobilities His material was extracted by potassium carbonate/potassium chloride mixture, the alkaline pH of which was commented on by Dr. Meyer.

DR. MUIR said that alkali brought about a lowering of viscosity, and because of this she had used the method of Blix and Snellman since it was the only method which she could find in the literature which made no use of alkali; and, in reply to a question by Dr Neuberger, indicated that this was probably the reason why the majority of the material she had extracted occurred in the combined form whereas other workers, using other methods, apparently as gentle, obtained polysaccharide with very low nitrogen content.

DR SNELLMAN pointed out the difficulty of securing complete separation from protein by the Sévag technique, and drew attention by analogy to the attempted separation of intact nucleo-protein

DR. PARTRIDGE felt that the very methods employed for the separation of the protein from the C.S A might also remove the mucoid material

In answer to a question by Dr. Meyer, DR MUIR stated that she did not think chondrosin would stand up to the hydrolytic conditions employed but the two sugar spots on her chromatogram both gave the Elson and Morgan reaction

DR. MEYER drew attention to the work of Schubert in which the ex- [illegible]

[illegible]

C.S.A. with distilled water They had often obtained nitrogen and [illegible]

the Sévag technique and adsorption on Lloyd's reagent and ion-exchange resins.

DR. MUIR stated that instead of decarboxylation measurements she had estimated the uronic acid carboxyl groups by difference between the sodium and sulphate analyses.

In reply to a question by Dr. Gross, DR MUIR stated that she could not find any tyrosine by ultraviolet absorption but agreed that it might have been destroyed during hydrolysis, especially in view of Dr. Gross's statement that tyrosine was considerably destroyed in the presence of large amounts of polysaccharides at acid pH.

DR. CONSDEN and DR. PARTRIDGE drew attention to the difference between Dr. Muir's material and that prepared respectively by Schubert and by Partridge. The former found 18 per cent of tyrosine and the latter, in the protein extracted from the complex, a tyrosine content of 4 per cent

In answer to a question by Dr. Meyer regarding the nature of the linkage between the polysaccharide and the protein, DR. MUIR stated that it was broken by decinormal alkali and hence one naturally thought in terms of an ester.

THE ARCHITECTURE OF THE COLLAGEN FIBRIL

R. REED

The first model for the configuration of the polypeptide chains in collagen was proposed almost twenty years ago (Astbury, 1940). By integrating the features of the wide-angle X-ray diffraction pattern with the chemical and physical data such as were then known, it afforded a most useful basis for workers in the field of collagen structure. This original model was essentially a straight single polypeptide chain, so that the complete fibril was considered to be built by the parallel arrangement of this chain. In recent years, however, it has gradually been realized that the polyptepide chains in the collagen fibril do not follow a straight course, on the contrary, ey appear to possess a helical configuration (Ramanchandran and artha, 1955; Ramanchandran, 1956; Rich and Crick, 1955; Cowan al., 1956). Thus workers in the field of X-ray diffraction now ccept the notion of a helical configuration, the protofibrillar unit 'the thinnest *filament* which carried the essential chemical and configurational structure of collagen', Bear, 1952), being regarded as a coiled-coil system of three polypeptide chains

It is pertinent, therefore, to discuss whether such a protofibrillar unit might lead to helical structures at higher levels of organization, such as are observed in the electron and in the ordinary light microscope. Most recent reviews of the architecture of the fibril (Bear, 1952; Grassmann, 1955), while accepting the helical structure of the protofibrillar unit, still account for the filaments and the complete fibril itself in terms of a parallel arrangement of this unit Thus Fig. 1 shows the various levels of organization within the fibril as suggested by Bear (1952). Furthermore, the cross-banded appearance, which is a characteristic feature of the collagen fibril, has been accounted for in various ways, all based on an essentially parallel ordering o the protofibrils Bear (1952), for example, considers that it is deter mined by the degree of perfection in which the protofibrils ar organized together sideways. In the interband regions, the prot fibrils are packed in almost perfect array, whilst in the band regior the packing is less perfect. Other workers, however (Grassmar

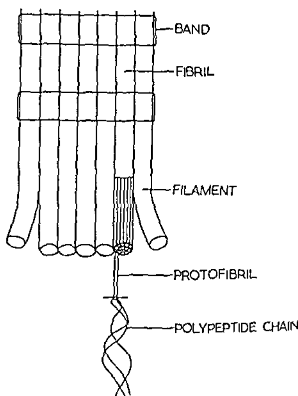


FIG 1
Scheme showing various levels of structure within the collagen fibril

1955), have suggested that the bands arise when certain regions of the protofibrils fold back upon themselves or when they overlap in special ways.

Various electron microscopists, however, have drawn attention to the peculiar helical forms which the collagen *fibril* may assume under a variety of experimental conditions (Wyckoff, 1949; Reed and Rudall, 1948; Swerdlow and Stromberg, 1955; Reed, Wood and Keech, 1956). The grinding of collagen fibrils in a Waring blendor, an operation frequently carried out in electron microscopic preparations, may lead to the formation of tactoidal structures, which appear to follow a helical course around the fibril axis (Keech, 1955; Swerdlow and Stromberg, 1955). Their formation seems to involve a loosening of the fibril along a helical path, so that the tactoids

appear as quite distinct structures (Fig. 2). Similar helical tactoids [illegible] collagen (Nutting [illegible]

[illegible]

helical tactoids has no preferred direction. Tactoids with both left- and right-hand spiral directions occur with approximately equal frequency, whilst sometimes both left- and right-hand spirals may be observed within the *same* fibril (Fig. 3). Tactoidal structures are often encountered during studies of the enzymatic digestion of collagen fibrils and also in the reconstitution of highly organized structures from solutions of material extracted by various chemical [illegible]

[illegible] ichthyocol collagen in acetic acid are precipitated by the addition of glycoprotein. Whether such tactoids, however, were originally arranged in a helical manner within the fibril, still needs further investigation

The helical tactoids can undergo subdivision into smaller structures (filaments), along lines which again follow a spiral course. As such subdivision occurs, the fibril appears to be composed of *two* sets of filaments, which cross each other in spiral fashion (Fig. 4) [illegible]

collagen which is [illegible]
(Reed and Ruda
cross-helical fash[illegible]
already mention[illegible]
right-hand spirals may be observed within one and the same fibril. It is important to note also that the formation of helical tactoids and of cross-helical arrays of filaments appears to involve a loosening of certain filaments in the intact fibril *along a spiral line which crosses the* [illegible]

(*a*) helical tactoids may be produced by the loosening of the fibril

(*b*) up,

(*c*) when helical tactoids and cross-helical arrays of filaments are produced, there is no great reduction in fibril or in filament length;

and these conditions are difficult to satisfy if the filaments are arranged in parallel fashion.

There are various ways of accounting for the helical structures manifested by the collagen fibril. Perhaps the most simple is as follows: Let us suppose that the *protofibrils,* the coiled-coil system of three polypeptide chains as indicated by the X-ray diffraction studies, are twisted together to form *filaments.* The *filaments* are then organized together in a steep, spiral fashion to form *two* independent identical *strands.* The two *strands* then spiral around each other to make up the complete fibril. Further, if in the normal intact fibril, the interspiralling of the two strands is steep, then all the filaments would lie very nearly parallel to the fibril axis, as indicated by the X-ray diffraction patterns (see Fig. 5).

Regarding the organization of the protofibrils into filaments, the electron microscope can provide little information, since the study of this level of structure demands a high degree of resolution which is beyond the scope of most present-day instruments Since, however, the protofibrils appear to possess a coiled configuration, it is likely that they do in fact twist together in forming the filaments The filaments on the other hand appear to be organized in very steep spirals when forming the strands.

It should be noted that certain authors have considered that the bands themselves follow a spiral path around the fibril axis (Swerdlow and Stromberg, 1955). Even if this disposition of the bands were substantiated, the spiral paths followed by the filaments appear to lie in directions quite distinct from that followed by the bands.

On the basis of the proposed model, the helical tactoids could arise by the loosening of *certain* filaments from their connections with their neighbours in the strands. The subdivision of the helical tactoids into thinner structures (and ultimately into filaments),

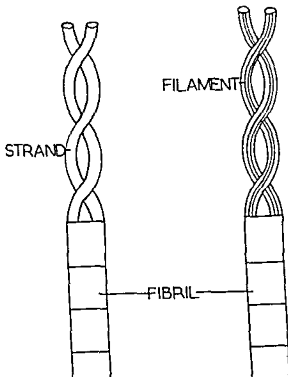


Fig 5
The suggested scheme for the architecture of the collagen fibril, based on the spiralling of two strands.

identical composition, it is difficult to explain the regularity in size of the helical tactoids and their regular subdivision into filaments.

whether better agreement with the X-ray diffraction data would come not from *one*, but from a *series* of protofibrillar units, each based on different numbers and types of polypeptide chains, remains

the result of a preferred manner of spreading of shrinkage along fibrils, possibly related, for example, to the disposition of molecules within the fibrils. Molecular ends are likely to be arranged in staggered, perhaps helical, fashion along the fibrils because the molecules have lengths which are about four times the normal macroperiod.

DR. REED gave three more pieces of evidence in favour of his observations: (1) tendon collagen gives even better spiral structures than skin collagen; (2) Dr. Grassmann showed a picture of a procollagen fibril in which there existed two strands which appeared to spiral around one another, and (3) Dr. Fitton Jackson also showed fibrils in cross-section which showed localization of filaments into definite strands within the fibril.

detracted from Dr. Reed's contention

DR. REED also drew attention to the fact that degradation studies, especially with enzymes, produced structures which were tactoidal in nature, and Dr. Gross's tropocollagen aggregates in this form as well

DR. GROSS felt that this was inconclusive since any rigid rod-like structure such as tobacco mosaic virus could aggregate in the form of tactoidal bundles.

In answer to a question by Dr. Fitton Jackson, DR. REED stated that they had no quantitative figures for the incidence of spiral or tactoidal structures in their material

DR. FITTON JACKSON inquired how Dr. Reed envisaged the tactoidal structures appearing from the spirals. To which he replied that he had not suggested that this occurred, merely that the spiral represented the initial stage of the phenomenon and that the fibres showed lines of weakness along the spiral. Referring to the last illustration in Dr. Reed's paper, DR.

areas where the helically wound filaments either crossed or lay

one another. In answer to a question by DR VAN DEN HOOFF who found it difficult to correlate the birefringence with such a structure, DR. REED pointed out that the effects concerned were only observed in treated fibres when the birefringence would anyway be impaired.

of tactoids in the colla-

pack-

ecific

points; to which Dr. Reed replied that this indeed had been the very point he wished to make.

have been given reasonably satisfactory interpretation in terms of variations in order and distortion occurring periodically along the fibrils. The fanning does, however, show some resemblance to the diffraction expected of gross helical twists, but the X-ray diagram on this basis would indicate a coil pitch equal to one macroperiod, the twists of Dr. Reed's

angles of Dr. Reed's fibrils with the X-ray 'fanning angle', which departs about 24° from the fibrillar axis or rises at about 66° to the equatorial (cross-sectional) plane.

Dr. Reed replied that the steepest angle of the spiral was about 70°.

regions in the fibril were more sensitive to shrinkage than others.

AMINO-ACID SEQUENCES OF COLLAGEN

W. GRASSMANN, K. HANNIG, H. ENDRES AND A. RIEDEL

The highly differentiated fine structure of collagen (Hall, 1942; Wolpers, 1943, 1944; Schmitt, 1948; Grassmann, 1952; Hofmann, 1952; Nemetschek, 1955) and procollagen (Kuhn *et al.*, 1956) visible in the electron microscope indicates that there is a relation between the molecular structure of these fibrous proteins and their electron microscopic picture. In order to gain an insight into the amino-acid sequence of collagen and pro-collagen, we have begun to separate the degradation peptides, obtained by digestion with crystalline trypsin, by means of electrophoretic and chromatographic methods and to study their chemical constitution.

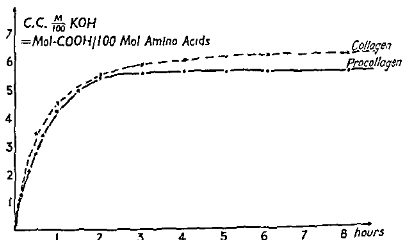


FIG 1
Tryptic digestion of
X---X Collagen and
·——— Procollagen



Fig 2
Human dermal collagen fibrils after fragmentation in a Waring blender Arrows show the helical tactoids.

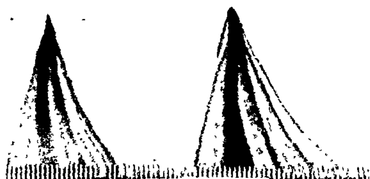


FIG 2
Electrophoretic separation patterns after tryptic degradation at pH 4.9 of
(a) collagen and
(b) procollagen

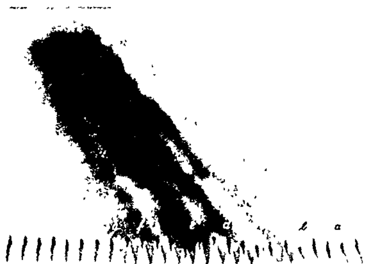


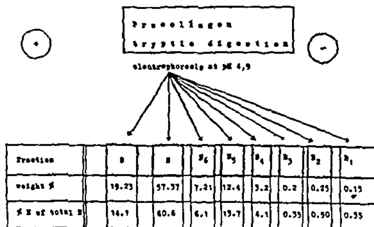
FIG 4
Further separation of the neutral fraction by electrophoresis at pH 2.3



Fig 5
Further separation of the basic fraction B_1 in 4 subfractions at pH 2 3

has a complicated composition. The separation of such complicated mixtures of peptides having such great chain lengths is by no means easy. Among other things, loose addition compounds of such polypeptides are apparently sometimes formed whose complete separation cannot be accomplished in one single operation. We have found the continuous electrophoresis (Grassmann and [illegible]

This and the quantitative amino-acid analysis indicate that there is a similar correspondence in the case of the amino acid sequences. As to procollagen, it can be purified by repeated recrystallization. The separation pattern is sharper which indicates that this material is of a more homogeneous nature. We have therefore mainly used such recrystallized procollagen in our experiments. Quantitative checks were made at all stages of the separations. Fig 3 shows a schematic



Fraction		S		N	S_6	N_5	B_4	B_3	B_2	B_1
weight %		19.23		57.37	7.21	12.4	3.2	0.2	0.25	0.15
% N of total N		14.7		60.6	6.1	13.7	4.1	0.33	0.50	0.33

FIG 3

Schematic diagram showing the electrophoretic isolation process of procollagen-peptides resulting from tryptic cleavage.

S = Acid fraction, N = neutral fraction, B = basic fractions.

diagram of the separation process and the yields obtained thereby.

Only very few of the fractions resulting from a single separation process at pH 4 9 are homogeneous. Most of them can be further

X

TABLE I

THE AMINO-ACID COMPOSITION OF THE HOMOGENEOUS PEPTIDES OBTAINED BY ELECTROPHORETIC SEPARATION OF THE TRYPTIC HYDROLYSATES

Fraction		polar								non-polar		
		B_1	B_{6a}	S_a	B_3	B_{6b}	S_b	S_d	S_e	B_{6c}	B_{6d} II	S_f
Yield (effect)	weight (per cent)	0,15	0,03	0,20	0,20	0,53	0,3	0,75	2,6	0,52	0,8	1,6
Total amount approx	weight (per cent) per cent N	0,15 0,3	0,07 0,1	0,35 0,33	0,2 0,35	1,20 1,3	0,75 0,77	1,65 1,65	4,7 4,7	1,3 1,4	3,5 3,4	3,15 3,05
Total N (per cent)		29,5	22,1	14,2	26,2	16,0	15,4	15,3	15,2	15,9	14,5	14,3
Gly		1	1	5	5	6	14	12	24	10	6	14
Ala		—	—	—	—	3	5	5	12	5	3	3
Val		—	—	—	—	1	1	1	1	1	1	—
Leu+ILeu		—	—	—	—	2	1	1	2	4	1	1
Phe		—	—	—	—	—	—	—	1	—	—	1
Ser		—	—	—	—	—	1	1	3	—	1	[illegible]
Thr		—	—	1	—	—	1	—	—	2	[illegible]	[illegible]
Tyr		—	—	—	—	—	—	—	—	[illegible]	[illegible]	[illegible]
Met		—	—	—	—	—	—	—	[illegible]	[illegible]	[illegible]	[illegible]

TABLE I, *continued*

	polar								*non-polar*		
Fraction	B_1	B_{2a}	S_a	B_3	B_{4b}	S_b	S_d	S_e	B_{4c}	B_{4d} II	S_f
Pro	—	—	—	—	1	5	6	11	5	2	10
Hypro	—	—	—	—	1	4	4	7	3	3	5
Asp	—	—	2	—	1	3	3	4	—	—	2
Glu	—	1	3	—	1	5	4	8	—	1	2
Lys	—	—	—	1	1	2	1	2	—	1	—
Hylys	—	—	1	—	1	—	—	—	1	—	—
His	1	—	—	—	1	—	—	—	2	—	—
Arg	1	1	1	4	1	1	1-2	3	1	1	—
Amide-N	—	—	—	—		(1)	(2)	(4)	—	—	—
Chain length	3	3	13	10	20	44	39-40	79	33	20	43-44
Glycine (mol. per cent)	33,3	33,3	38,5	50,0	30,0	31,8	30,8	29,2	30,3	30,0	32,6
Pro+Hypro (mol per cent)	—	—	—	—	10,0	20,5	25,5	22,8	18,2	25,0	34,8
Diamino acids (mol. per cent)	66,6	33,3	15,4	50,0	20,0	9,1	5-8	6,4	15,2	10,0	—
Dicarboxylic acids (mol. per cent)	—	33,3	38,5	—	10,0	18,2	18,0	15,2	—	5,0	9,3
Sum of diamino and dicarboxylic amino-acids	66,6	66,6	53,9	50,0	30,0	27,3	23-26	21,6	15,2	15,0	9,3

TABLE I

THE AMINO-ACID COMPOSITION OF THE HOMOGENEOUS PEPTIDES OBTAINED BY ELECTROPHORETIC SEPARATION OF THE TRYPTIC HYDROLYSATES

		polar							*non-polar*			
Fraction		B_1	B_{6a}	S_a	B_2	B_{5b}	S_b	S_d	S_e	B_{4c}	B_{6d} II	S_f
Yield (effect)	weight (per cent)	0,15	0,03	0,20	0,20	0,53	0,3	0,75	2,6	0,52	0,8	1,6
Total amount approx	weight (per cent)	0,15	0,07	0,35	0,2	1,20	0,75	1,65	4,7	1,3	3,5	3,15
	per cent N	0,3	0,1	0,33	0,35	1,3	0,77	1,65	4,7	1,4	3,4	3,05
Total N (per cent)		29,5	22,1	14,2	26,2	16,0	15,4	15,3	15,2	15,9	14,5	14,3
Gly		1	1	5	5	6	14	12	24	10	6	14
Ala		—	—	—	—	3	5	5	12	5	3	3
Val		—	—	—	—	1	1	1	1	1	1	—
Leu+ILeu		—	—	—	—	2	1	1	2	4	1	1
Phe		—	—	—	—	—	—	—	1	—	—	1
Ser		—	—	—	—	—	1	1	3	—	1	2-3
Thr		—	—	1	—	—	1	—	—	2	—	1
Tyr		—	—	—	—	—	—	—	—	—	—	2
Met		—	—	—	—	—	—	—	1	—	—	—

Table I, continued

	polar								non-polar		
Fraction	B_1	B_{4a}	S_a	B_3	B_{3b}	S_b	S_d	S_e	B_{4c}	B_{4d} II	S_f
Pro	—	—	—	—	1	5	6	11	3	2	10
Hypro	—	—	—	—	1	4	4	7	3	3	5
Asp	—	—	2	—	1	3	3	4	—	—	2
Glu	—	1	3	—	1	5	4	8	—	1	2
Lys	—	—	—	1	1	2	1	2	—	1	—
Hylys	—	—	1	—	1	—	—	—	2	—	—
His	1	—	—	—	1	—	—	—	2	—	—
Arg	1	1	1	4	2	2	1–2	3	1	1	—
Amide-N	—	—	—	—		(1)	(2)	(4)	—	—	—
Chain length	*3*	*3*	*13*	*10*	*20*	*44*	*39–40*	*79*	*33*	*20*	*43–44*
Glycine (mol. per cent)	33,3	33,3	38,5	50,0	30,0	31,8	30,8	29,2	30,3	30,0	31,6
Pro+Hypro (mol per cent)	—	—	—	—	10,0	20,5	25,5	22,8	18,2	25,0	34,8
Diamino acids (mol per cent)	*66,6*	33,3	15,4	50,0	20,0	9,1	5–8	6,4	15,2	10,0	—
Dicarboxylic acids (mol. per cent)	—	33,3	38,5	—	10,0	18,2	18,0	15,2	—	5,0	9,3
Sum of diamino and dicarboxylic amino-acids	66,6	66,6	53,9	50,0	30,0	27,3	23–26	21,6	15,2	15,0	9,3

TABLE I

THE AMINO-ACID COMPOSITION OF THE HOMOGENEOUS PEPTIDES OBTAINED BY ELECTROPHORETIC SEPARATION OF THE TRYPTIC HYDROLYSATES

Fraction		*polar*								*non-polar*		
		B_1	B_{4a}	S_a	B_2	B_{4b}	S_b	S_d	S_e	B_{4c}	B_{4d} II	S_f
Yield (effect)	weight (per cent)	0,15	0,03	0,20	0,20	0,53	0,3	0,75	2,6	0,52	0,8	1,6
Total amount approx	weight (per cent)	0,15	0,07	0,35	0,2	1,20	0,75	1,65	4,7	1,3	3,5	3,15
	per cent N	0,3	0,1	0,33	0,35	1,3	0,77	1,65	4,7	1,4	3,4	3,05
Total N (per cent)		29,5	22,1	14,2	26,2	16,0	15,4	15,3	15,2	15,9	14,5	14,3
Gly		1	1	5	5	6	14	12	24	10	6	14
Ala		—	—	—	—	3	5	5	12	5	3	3
Val		—	—	—	—	1	1	1	1	1	1	—
Leu+ILeu		—	—	—	—	2	1	1	2	4	1	1
Phe		—	—	—	—	—	—	—	1	—	—	1
Ser		—	—	—	—	—	1	1	3	—	1	
Thr		—	—	1	—	—	1	—	—	2		
Tyr		—	—	—	—	—	—	—	—			
Met		—	—	—	—	—	—					

TABLE I, *continued*

	polar								*non-polar*		
Fraction	B_1	B_{2a}	S_a	B_3	B_4b	S_b	S_d	S_e	B_4c	B_4d II	S_f
Pro	—	—	—	—	1	5	6	11	3	2	10
Hypro	—	—	—	—	1	4	4	7	3	3	5
Asp	—	—	2	—	1	3	3	4	—	—	2
Glu	—	1	3	—	1	5	4	8	—	1	2
Lys	—	—	—	1	1	2	1	2	—	1	—
Hylys	—	—	1	—	1	—	—	—	2	—	—
His	1	—	—	—	1	—	—	—	2	—	—
Arg	1	1	1	4	1	2	1-2	3	1	1	—
Amide-N	—	—	—	—		(1)	(2)	(4)	—	—	—
Chain length	3	3	13	10	20	44	39-40	79	33	20	43-44
Glycine (mol per cent)	33,3	33,3	38,5	50,0	30,0	31,8	30,8	29,2	30,3	30,0	32,6
Pro+Hypro (mol per cent)	—	—	—	—	10,0	20,5	25,5	22,8	18,2	25,0	34,8
Diamino acids (mol. per cent)	*66,6*	*33,3*	*15,4*	50,0	20,0	*9,1*	*5-8*	*6,4*	15,2	10,0	—
Dicarboxylic acids (mol. per cent)	—	*33,3*	*38,5*	—	*10,0*	*18,2*	*18,0*	*15,2*	—	*5,0*	*9,3*
Sum of diamino and dicarboxylic amino-acids	66,6	66,6	53,9	50,0	30,0	27,3	23-26	21,6	15,2	15,0	9,3

TABLE I

THE AMINO-ACID COMPOSITION OF THE HOMOGENEOUS PEPTIDES OBTAINED BY ELECTROPHORETIC SEPARATION OF THE TRYPTIC HYDROLYSATES

Fraction		polar								non-polar		
		B_1	B_{4a}	S_a	B_a	B_ab	S_b	S_d	S_e	B_{4c}	B_{6d} II	S_f
Yield (effect)	weight (per cent)	0,15	0,03	0,20	0,20	0,53	0,3	0,75	2,6	0,52	0,8	1,6
Total amount approx.	weight (per cent)	0,15	0,07	0,35	0,2	1,20	0,75	1,65	4,7	1,3	3,5	3,15
	per cent N	0,3	0,1	0,33	0,35	1,3	0,77	1,65	4,7	1,4	3,4	3,05
Total N (per cent)		29,5	22,1	14,2	26,2	16,0	15,4	15,3	15,2	15,9	14,5	14,3
Gly		1	1	5	5	6	14	12	24	10	6	14
Ala		—	—	—	—	3	5	5	12	5	3	3
Val		—	—	—	—	1	1	1	1	1	1	—
Leu+ILeu		—	—	—	—	2	1	1	2	4	1	1
Phe		—	—	—	—	—	—	—	1	—	—	1
Ser		—	—	—	—	—	1	1	3	—	1	2–3
Thr		—	—	1	—	—	1	—	—	2	—	1
Tyr		—	—	—	—	—	—	—	—	—	—	2
Met		—	—	—	—	—	—	—	1	—	—	—

TABLE I, continued

	polar									*non-polar*	
Fraction	B_2	B_{2a}	S_a	B_3	B_{3b}	S_b	S_d	S_e	B_{4c}	B_{5d} II	S_f
Pro	—	—	—	—	1	5	6	11	3	2	10
Hypro	—	—	—	—	1	4	4	7	3	3	5
Asp	—	—	2	—	1	3	3	4	—	—	2
Glu	—	1	3	—	1	5	4	8	—	1	2
Lys	—	—	—	1	1	2	1	2	—	1	—
Hylys	—	—	1	—	1	—	—	—	2	—	—
His	1	—	—	—	1	—	—	—	2	—	—
Arg	1	1	1	4	1	2	1-2	3	1	1	—
Amide-N	—	—	—	—		(1)	(2)	(4)	—	—	—
Chain length	3	3	13	10	20	44	39-40	79	33	20	43-44
Glycine (mol. per cent)	33,3	33,3	38,5	50,0	30,0	31,8	30,8	29,2	30,3	30,0	32,6
Pro+Hypro (mol. per cent)	—	—	—	—	10,0	20,5	25,5	22,8	18,2	25,0	34,8
Diamino acids (mol. per cent)	66,6	33,3	15,4	50,0	20,0	9,1	5-8	6,4	15,2	10,0	—
Dicarboxylic acids (mol. per cent)	—	33,3	38,5	—	10,0	18,2	18,0	15,2	—	5,0	9,3
Sum of diamino and dicarboxylic amino-acids	66,6	66,6	53,9	50,0	30,0	27,3	23-26	21,6	15,2	15,0	9,3

separated into sub-fractions, either by electrophoresis at anoth value or by means of chromatography. Fig. 4, for example, s the further separation of the neutral fraction by electrophores pH 2.3. Fig. 5 shows the separation of the basic fraction B_1 into sub-fractions, also at a pH value of 2.3. We do not consider a f tion homogeneous until we have found that no further separation lectrophoresis is possible at three pH values (2.3; 4.9; 9.2). Mo ver, this criterion must apply in four different solvent mixtures hromatography. Finally, the quantitative amino-acid analys rassmann, Hannig and Plöckl, 1955) must result in a clear-cut an tegral ratio of all amino acids. These strict requirements whic herto have not been applied on ascertaining the constitution of teins and protein degradation products appear to be necessary in our opinion At present we have obtained about eleven peptides that fulfil such requirements.

Table I contains the actual yield of the individual peptides in per cent by weight and an estimate on the quantitative percentage in which they are contained in the degradation mixture. Since the purification process is at all stages checked by quantitative analyses, this estimate is fairly exact.

The accuracy of the analyses from which we obtain the stoichiometrical ratio of the amino acids may be illustrated by two examples, fraction S_4 and fraction B_{1e} (Table II).

Table III illustrates the determination of the constitution of Fraction B_1 which is homogeneous after the first separation. However, it is obtained in insignificant quantities only. The sequence is gly-arg-his (Grassmann, unpublished).

Glycine is present in fairly uniform quantities in all fractions as shown in Table I. It usually amounts to one-third of the total amino acids, and only in two cases – the quantitatively rather small fraction S_4 and B_3 – did we find values of 38 and 50 mol per cent of the total amino acids respectively.

If the peptides so far obtained are listed in the order of their molecular contents of highly polar amino acids, i.e. of the sum of diamino and dicarboxylic acids, on the one hand, and in the order of their contents of the amino acids proline and hydroxyproline, on the other hand, we find that if the order of the one group of amino acids increases, in the other group it decreases. Split peptides with a high content of polar amino acids contain little or no proline and hydroxyproline and vice versa. These findings alone render the

TABLE II

STOICHIOMETRICAL RATIO OF THE AMINO ACIDS OF FRACTIONS S_a AND B_{4c}

	S_a		B_{4c}	
	Total N 14,3 per cent *Amide N 0,05 per cent*		*Total N 14,3 per cent* *Amide N 0,00 per cent*	
	Mole ratio found	*Mole ratio rounded*	*Mole ratio found*	*Mole ratio rounded*
Gly	4,95	5	10,3	10
Ala	—	—	5,1	5
Val	—	—	1,15	1
Leu+Ileu	—	—	3,8	4
Phe	—	—	—	—
Ser	—	—	—	—
Thr	0,97	1	1,96	2
Tyr	—	—	—	—
Meth	—	—	—	—
Pro	—	—	2,95	3
Hypro	—	—	3,40	3
Asp	2,04	2	—	—
Glu	3,03	3	—	—
Lys	—	—	—	—
Hylys	1,00	1	1,67	2
His	—	—	1,97	2
Arg	1,00	1	1,2	1
	12,99	13	33,5	33

TABLE III

DETERMINATION OF THE CONSTITUTION OF FRACTION B_1

FRACTION B1 (GLY-ARG-HIS)

	*Per cent of total N**	*Mole per cent*
Glycine	12,7	33,6
Histidine	37,8	33,3
Arginine	49,7	33,1
	100,2	100,0

N-terminal amino acid† Glycine
C-terminal amino acid‡ Histidine

* W. Grassmann, K. Hannig and M. Plöckl, *Hoppe-Seyl. Z. physiol. Chem.*, 305, 21 (1956)
† F. Sanger, *Biochem. J.*, 39, 507 (1945)
‡ W. Grassmann, H. Hörmann and H. Endres, *Ber.*, 86, 1477 (1953)

theory of Bergmann and Niemann (1936) improbable. assumed a regular recurrence of tripeptide units consisting of cine, 1 proline or hydroxyproline and one other amino aci conformity with other authors (Schroeder *et al.*, 1953, 1954; Kr *et al.*, 1953, 1955; Bear, 1952) the results rather indicate an alte tion of non-polar areas that are rich in proline and hydroxyprol and of polar ranges poor in amino acids which might account the dark and light bands (Schroeder *et al.*, 1954).

In order to gain further insight on the proline and hydrox proline containing sequences, we have studied initially the fractio S_f which has the highest content of amino acids of all fractions. A a pH value of 4.9 it migrates slowly towards the anode, and at pH 2.3 its migration path shows the least deviation of all acid peptides towards the cathode. This, of course, facilitates its isolation. The actually isolated quantity of this fraction is equivalent to 1.55 per cent of the total nitrogen, and 1.6 per cent of the weight of procollagen. Its actual proportion in the mixture is about 3 per cent. The fraction makes up almost 5 per cent of the total content of proline and hydroxyproline.

TABLE IV

QUANTITATIVE AMINO-ACID ANALYSIS OF THE FRACTION S_f

Total N 14,3 per cent
Amide N 0,05 per cent

	Per cent of total N		Mole ratio found		Mole ratio rounded
	I	II	I	II	
Gly	31,40	31,90	14,00	13,95	14
Ala	6,53	6,18	2,92	2,70	3
Leu+Ileu	1,68	1,95	0,75	0,85	1
Phe	2,44	2,63	1,09	1,15	1
Ser	6,05	5,15	2,70	2,25	2-3
Thr	1,88	1,83	0,84	0,80	1
Tyr	4,10	2,97	1,83	1,30	2
Pro	22,9	23,40	10,2	10,2	10
Hypro	11,42	11,35	5,2	4,95	5
Asp	4,70	4,74	2,05	2,07	2
Glu	4,36	4,70	1,95	2,05	2
	97,46	96,80	43,53	42,27	43-44

I = Hydrolysis with 6 n HC , 24 hours at 100° C
II = Hydrolysis with 6 n HC , 48 hours at 100° C.

The amino-acid analysis (Table IV) shows the complete absence of basic amino acids and of amide nitrogen. The chain length established by the amino-acid analysis is 43 to 44 amino acids, which is equivalent to a molecular weight of 3900 to 4000. Glycine, and only glycine, is found in the N-terminal residue by the Sanger method and only aspartic acid in the C-terminal residue by the reduction method and by the use of carboxypeptidase (Grassmann, Hormann and Endres, 1953, 1954, 1955). The yield of aspartic acid established by the reduction method was found to be equivalent to 0 7 mol for 43 amino acids.

The photometrical determination in the case of the DNP-derivative showed a molecular weight of 3717 for the free peptide. The potentiometric titration of the DNP-derivative shows a carboxyl equivalent of 727. This corresponds to a molecular weight of 3635 [illegible]

to the chromatographic and electrophoretic examination.

Almost exactly one-third, that is to say 14, of the total amino acids are glycine, another one-third or 15 residues are proline and hydroxyproline. The remaining 15 to 16 residues are other amino acids.

Thus, as far as its composition is concerned, the fraction might very well be in line with the conception of Bergmann and Niemann. However, the examination of the amino-acid sequence led to results that entirely contradict any such assumption
[illegible] 2 mol of aspartic acid (F[illegible] phenyl-alanine follow ea[illegible]ne nor hydroxyproline are split off, however. It has been shown that C-terminal leucine is much more rapidly split off by carboxypeptidase than aspartic acid (Neurath and Schwert, 1950). The sequence in the C-terminal must therefore be leu-asp-asp-COOH. If the sequence were asp-leu-asp-COOH, leucine would have to be split off much more quickly than the second mol of the aspartic acid. An analogous consideration holds true in the case of phenyl-alanine and glutamic acid. Hence it is highly probable that the sequence in the C-terminal residue is

. . . phe-glu-leu-asp-asp-COOH.

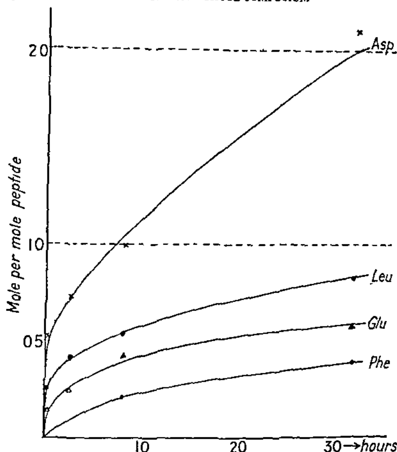


Fig 6
Quantitative determination of the amino acids split off from the procollagen fraction Sf by carboxypeptidase after different times

At any rate, it can be said with certainty that proline and hydroxyproline are not among the first five amino acids in the C-terminal residue.

Hydrolysis with aminopolypeptidase from yeast (Grassmann,
[illegible] threonine and alanine
[illegible] present only once accord-
[illegible] the C-

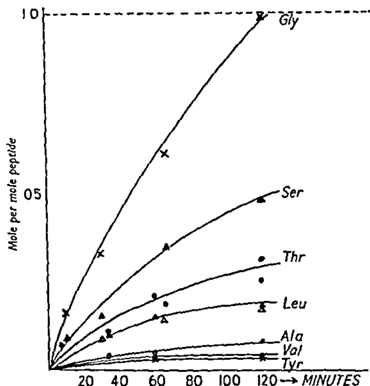


Fig 7
Quantitative determination of the amino acids split off from the procollagen fraction S_f by aminopeptidase after different times

The partial hydrolysis of the DNP-derivative resulted in DNP-peptides which, apart from terminal glycine, contained serine, threonine, alanine, further glycine, and again small quantities of leucine. [illegible]

determination of the amino-acid sequence in the N-terminal residue. It is certain, however, that glycine, serine, alanine, threonine and one or two more glycine residues must be among the first six amino

acids from the N-terminal residue, whereas proline and hydroxyproline cannot be among the first six.

The amino acids from the middle of the peptide are found among the peptides obtained as DNP-free peptides in the partial hydrolysis of the DNP-derivative. They contain considerable quantities of proline, hydroxyproline and glycine, and also serine, alanine and tyrosine. The constitution of the peptide can thus be set down as follows:

FORMULA I

CONSTITUTION OF THE PEPTIDE Sf

H_2N-gly-(ser, thr, leu, ala, gly)-

N-terminal section

[10 pro, 5 hypro, 12 gly, 2 ala, 1-2 ser, 2 tyr, 1 glu]

centre section

-phe-glu-leu-asp-asp-COOH

C-terminal section

If it is assumed that glutamic acid and tyrosine are actually contained in the N-terminal or C-terminal section – our experiments do not exclude this possibility – it follows that the middle part must be made up of 10 prolines and 5 hydroxyprolines on the one hand, and about the same number of very simple amino acids, namely 12 glycine, 2 alanine and 1-2 serine, on the other.

W. A. Schroeder *et al.* (1953, 1954) and Kroner *et al.* (1953, 1955) earlier, found gly-pro, hypro-gly and gly-(pro, hypro)-gly as disintegration products by means of partial hydrolysis with acids. These authors are of the opinion that the tetra-peptide units gly-pro-hypro-gly are essential structural elements of the crystalline parts of the fibril. The findings of Gustavson (1953), who showed that the thermo-stability of collagenous fibrils increases with their proline and hydroxyproline contents, are also on the same line.

Our findings support the conception of Schroeder and apparently prove the existence of a non-polar part containing about 30 amino acids whose sequence is very similar to the one assumed by Schroeder.

We should like to postpone a more thorough discussion of our findings relating to the structure models recently suggested by Ramachandran and Kartha (1955), Rich and Crick (1955) and

Cowan *et al.* (1955) until after we have fully explored the amino-acid sequence of the middle part. At any rate, its length of about 30 residues would correspond to one turn in the last model proposed by Ramachandran and it would be of a magnitude that could correspond to a single light band.

For any further determination of the collagen structure it will be of particular significance whether the *entire* proline and hydroxy-proline is present in aggregated groups of the type shown and whether it will be possible to establish a relation of this sequence with the electron microscope picture.

GROUP DISCUSSION

... the most recently proposed 3-chain coiled-
... the typi-
... ather than
4-residue ... l gly-pro-
hypro sequences, or variations therefrom ... of the last
...
... sequence G-R-R,
... *al*) G must be
... *can* be amino
... discussion of the
... , he pointed out
... be *in* contradic-
... glycine-proline-
hydroxy-proline and five other ... glycine-glycine-proline (where glycine represents a 'glycine-like' residue) would be in satisfying agreement with his analytical results

DR BEAR observed that at the C-terminal end of the S_F peptide glycine did not occur in every third position.

DR. GRASSMANN said that this was really a surprising result. His other results show rather good agreement with the assumption of a regular ... residues
... will be important
... postulated, say
... the fibril, or
between the main length of the ... endages. He then showed a photograph of the 3-chain coiled-coil type of structure now favoured from physical evidence for the major portion of the collagen molecule.

In reply to Dr. Orekhovitch, Dr Grassmann summarized the procedure for determining the C-terminal sequence with carboxypeptidase. [illegible]

trypsin could be inhibited by di-isopropyl fluorophosphate. Dr. Orekhovitch said that even after ten recrystallizations carboxypeptidase still contained proteinase which could not be inhibited by di-isopropyl fluorophosphate and that he preferred chemical methods. Dr. Grassmann replied that a reduction method for determining the C-terminal residue gave results in quantitative agreement with the enzymic method but pointed out that there was no reliable chemical method for determining *stepwise* the C-terminal *sequence*.

Dr. Snellman suggested that if the trypsin used was pure then the fact *that the* S_f *peptide contained no lysine or arginine means that* S_f *is a* terminal peptide Dr Grassmann said this was possible and added that other peptides isolated did contain C-terminal lysine and arginine. Some peptides have been isolated where this is not true.

In reply to Dr Neuberger, Dr. Grassmann said that he had used recrystallized trypsin and that several samples gave the same results as regards the end point of the reaction Traces of other enzymes might be present but he did not think this affected his main conclusions. In reply to Dr Consden, Dr Grassmann said that trypsin was used because its reaction with the substrate had a well-defined end point. The collagen was heated to its shrinkage temperature before being treated with trypsin.

THE COMPOSITION OF BANDS AND INTERBANDS OF COLLAGEN FIBRILS[1]

RICHARD S. BEAR AND RICHARD S. MORGAN

Investigators of connective tissue most often encounter the collagenous component as macroscopically or microscopically visible *fibres* of diameters 2 to 200 μ. These are formed of sub-microscopic *fibrils* whose diameters may range from a few hundred to several thousand Ångstrom units. In some circumstances it is convenient to consider also subfibrillar units, *filaments* and *protofibrils* (Schmitt, Hall and Jakus, 1942; Bear, 1952), which result from successively finer stages of fibrillar subdivision.

Experimental demonstration of the existence of filaments as significant natural fibrillar units in collagen is still uncertain, because some degree of accidental longitudinal cleavage within fibrils is to be expected during manipulative procedures. Protofibrils were originally 'the unit columnar arrays which, when associated laterally,

[illegible]

involved in fibrillar formation.

The fibril, however, provides the unit of more direct interest in biological and medical problems. As a result of recent work the molecule is now well defined in several important respects, but fibrillar structure is much less well determined. This paper is devoted to the latter problem.

[illegible] Leaving [illegible] erimental [illegible] ie second aspect, related to longitudinal disposition of molecules.

[1] This work was supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and also in part by Research Grant A-901 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U.S. Public Health Service

One of us (Bear, 1952) has already reviewed preliminary ideas regarding the structural meaning of the bands and interbands of the fibrillar macroperiod, and here we shall revise and extend these ... in the light of new evidence. The development below is ... ands and interbands as ... along molecules and of molecules along the fibril.

EXPERIMENTAL ESTIMATION OF MATERIAL DISTRIBUTION ALONG FIBRIL AXES

... Grassmann and Hofmann (1955) and Burge and ...

sitometry of electronmicrographs. A disappointing ... kind of work, emphasized by the latter authors, is the variability of result obtained from one macroperiod or fibril to another. One may ... whether this great degree of variability still obtains in ...

ness and number of layer lines obtained. ... of interest to learn what small-angle intensity data can indicate about the distribution of matter along collagen fibrils.

The most direct procedure would be to determine what crystallographers call a Patterson projection, expressing the frequency with which vectors of different size connect parcels of electron density along the fibrillar axis. These were made early in this laboratory for ... phosphotungstate staining princip- ... iple of Bear, Bolduan and Salo, ... those expected between *a* and all other known bands, as judged from electron-optical measurements of band positions (Schmitt and Gross, 1948) seemed to dominate the Patterson plot. The result was not convincing, however, and was not published.

The chief difficulty with Patterson plots is that they lack resolution ... to separate interband vectors, probably because the ... sharp bands ... plotted in the ... Patterson ... uld a single

xtensive band, covering 0.46 to 0.47 of the macroperiod (Kaesberg nd Shurman, 1953; Tomlin and Worthington, 1956). This could [illegible] fraction, 0.53 to 0.54, [illegible] distinguish 'positive' [illegible] Both investigations [illegible] gree that dry collagen is less simply [illegible]

The Patterson plots are feasible because observed intensities of X-ray diffraction orders can be used alone. To obtain direct distributions of electrons (Fourier plots) one requires phases, as well as amplitudes (square roots of intensities), for the several orders. The phases are not experimentally determinable from the X-ray data alone. One can approach this problem in an indirect way by using models, derived from examination of electron micrographs or assumed from inspection of the small-angle diffraction data, to calculate phases, which can then be used with X-ray amplitudes to make the direct plots. This has been done by Tomlin and Worthington (1956), who, however, made the assumption, contradicted by electron optical information, that there are cross-sectional planes of symmetry in the fibril.

Recently, to facilitate this type of indirect approach, we have been using an optical diffraction apparatus designed by Dr. H. W. Wyckoff, to whom we are also indebted for suggestions of ingenious [illegible] along the way. In brief, we first design models [illegible] drawings of these are made and photographically [illegible] the resulting 'mask' is made to diffract monochromatic visible light (the green 'line' from a mercury arc), and the final optical diffraction pattern is analysed or compared directly with the appropriate X-ray diffraction pattern. Satisfactory models are then used for calculation of phases. The X-ray intensities employed are unpublished ones determined at various times for kangaroo tail tendon in this laboratory by Drs. O. E. A. Bolduan and T C. Furnas, Jr, in essential agreement with data given by Tomlin and Worthington (1956). Actually, the models themselves are satisfactory for most present purposes, and although quantitative calculations have been applied for comparison of intensities predicted by the models with observed ones, we rely here chiefly on photographic presentation of the results.

ELECTRON DISTRIBUTION ALONG MOIST AND DRY FIBRILS

Initially we determined arbitrarily to limit the resolution of our models to spacings (40 to 45 Å.) corresponding to the fifteenth diffraction order and approximating the electron-optical resolution of bands. Less than 1 per cent of diffracted energy in the small-angle meridional system occurs beyond the fifteenth order, and any attempts to draw significant conclusions about smaller detail (except at wide angles) would be fruitless at present. The models were then constructed as combinations of rectangular apertures, each with the dimension along the fibril axis equal to or greater than the resolution.

We first sought a simple way of representing band structure, based largely on data published by Schmitt and Gross (1948) and by Nemetschek, Grassmann and Hofmann (1955). Inspection of this information suggested that the following bands, at the fractional locations along the macroperiod given, would be suitable: *d*, 0 00 *e*, 0.16; a_1, 0.30; a_2, 0.39; b_1, 0.54; b_2, 0.64; *c*, 0.84. These wer selected as the most consistently observed, strongest bands, whic for simplicity could be given equal weight to represent the maj features of band structure. In the models these were given leng along the fibril axis equal to the resolution (0.067 of the mac period). It follows also, from diffraction theory, that separ apertures of this size, hence all of them, cannot contribute m diffracted radiation to the neighbourhood of the fifteenth orde is required by the X-ray diffraction data.

Fig. 1 shows a series of macroperiods containing only bands, a with the corresponding optical diffraction pattern. It is immed reassuring that the intensities of the orders beyond the fifth rese the corresponding area of the X-ray pattern for dry collage Fig 3), indicating that the chosen band model can appropriat used to account for most of this part of the diffraction field. I tant X-ray diffraction at the lower orders is, however, not acc for by the simple model

Inspection of the problem of how to introduce this lo scatter into the model shows that this can be done very si follows: a new, longer 'background' aperture must be int which in particular should not add intensity near the weak tenth diffraction orders. Diffraction theory readily shows can be accomplished if the length of the background

Model

Optical Diffraction

9

6

c

3

b_1^2

a_1^2

e

d

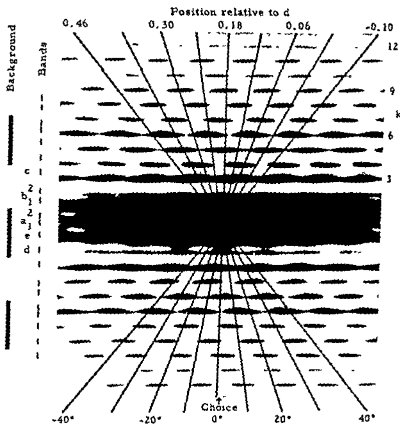


FIG 2

centre as origin Increase of the lateral separation between background and bands permits greater range of positional testing, as had been done prior to this final trial, whose best solution is indicated by 'choice'

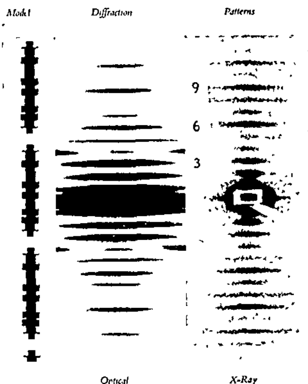


FIG. 3

Comparison of the model for the dry fibril with corresponding optical and X-ray diffraction. Bands are the extra widths applied to backgrounds with bands *d* and *e* at background edges. In this model background covers 0.78 of the macroperiod with centre at 0.62 relative to *d* and horizontal width 1.5 times that added by bands.

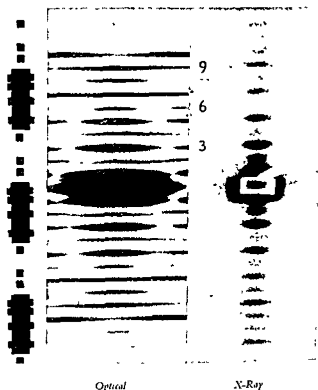


FIG 4

Comparison of the model for the moist fibril with corresponding optical and X-ray diffraction Here, background covers 0 54 of the macroperiod, with centre at 0 18 relative to *d* and horizontal width 2 5 times that added by bands

multiple of fifths of the macroperiod. Inspection of electron optical data suggested approximately four-fifths for background coverage and further refinements were canvassed as follows.

the optical method exemplified by Fig. 2. This figure is, however, for the more crucial similar problem encountered with wet fibrillar models. Background and band portions of the model were separated laterally and given different widths corresponding to weighting.

bands.

The solution finally selected for the model reproducing dry collagen diffraction is shown in Fig. 3. Note that optical and X-ray patterns should, in principle, be compared only directly at the individual line centres; the reasons for variations in horizontal line lengths are different in the two cases. The agreement is good, although doubtless it could be improved by incorporating further detail into the models, but this seems unwarranted at present

, one
have
but
it as

slightly denser levels than their surroundings, and located at essentially the same relative positions as in the dry situation. The pronounced alteration of small-angle X-ray order intensities (with odd orders strong, even ones weak relative to neighbours, which continues out to the eleventh order, *cf.* Fig. 4), suggests a background

maintaining moderately strong orders of indices 5, 7, 9 but not 11. It was also necessary to put a moderate amount of intensity into the

xactly
were
result,

shown in Fig. 4.

MAGNITUDES OF ELECTRON DENSITY VARIATIONS

In Fig. 5 the models thus far derived for the moist and dry fibrils are compared. Dotted lines indicate the result of using phases calculated from the models, along with X-ray amplitudes, to form Fourier plots of electron density projected on the fibrillar axis. It will be noted that the curve for dry collagen is very simila densitometer traces of Nemetschek, Grassmann and Hofm (1955, their Fig. 1) for kangaroo tendon, stained, however, w phosphotungstate.

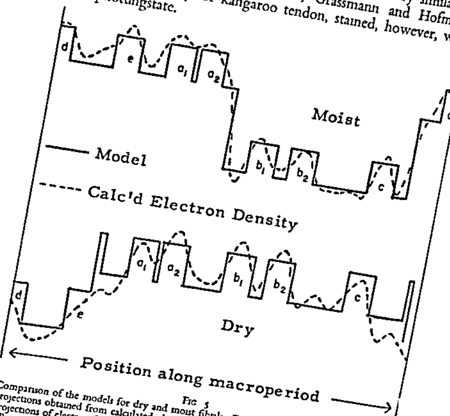


Fig 5

Comparison of the models for dry and moist fibrils. Dotted curves show electron density projections obtained from calculated phases and X-ray amplitudes. Ordinates express axial projections of electron density in arbitrary units One macroperiod is represented horizontally

In the following discussion it ... to realize that the ordi... s of Fig. 5 are in arbitrary ... one cannot at ... mate the absolute scale of ... the relative scal

the two plots. One may arrive at qualitative suggestions regarding this matter from consideration of what is known of the molecules involved in fibril formation.

Four separate lines of attack (Rich and Crick, 1955; Cowan, [illegible]

polypeptide configuration in native collagen, one expects the favoured kinds of 3-strand structure to extend throughout the molecular length, except when distortions are introduced as described below.

Electron-optical (Gross, Highberger and Schmitt, 1954) and physico-chemical (Boedtker and Doty, 1955) evidence also indicates that the collagen molecule is a thin particle (diameter 14 Å.) of great length (2500 to 3000 Å.). The molecular length is about four times the 600 to 700 Å. fibrillar macroperiod experienced with dry to moist native specimens as measured by X-rays It follows that some sort of extensive relative axial displacement of molecules, from exact transverse register of their ends, is necessary to explain development of a native fibrillar macroperiod shorter than individual molecular lengths. Evidence of the way this occurs may develop when small-angle, near-equatorial diffraction is more thoroughly explored [illegible]

importance to the problem of axial distribution of matter are his suggestions regarding 'interstitial' overlapping of molecular ends, or the alternative 'defects' arising where large gaps may be left between ends. Tomlin's bands and interbands arise from these end effects and individually extend over appreciable contiguous fractions of the macroperiod. They are not comparable to the narrower separable and distributed stratifications usually meant by these terms, being more like our backgrounds One of us (Bear, 1952) has suggested that the finer bands are caused by compositional differences, with residues of larger molal volumes preferentially congregated at bands.

Electron-optical replica studies show that moist fibrils are smoo (Gross and Schmitt, 1948), and essentially similar conclusions follo from studies of small-angle meridional line lengths (Bolduan an Bear, 1951). Molecular distortions leading to fibril corrugation ar largely 'ironed' out by moisture, leading to a more perfect structure (Rougvie and Bear, 1953). If equal numbers of molecules pass through equal cross-sections at all fibrillar levels, or even if overlaps or defects cause shifting of water around within the fibril, relatively small differences can be expected in electron density projected on the axis of a smooth fibril. We estimate about 3 to 4 per cent diff between interbands and bands, with the latter being the d assuming that the two differ only compositionally in an ext way. Molecular end effects could add to this difference a s amount.

Dry fibrils are, as is well known, corrugated by diametral va tions. Intrinsic material densities, assuming even extreme co positional differences between bands and interbands, can be show to be very little different from the average over-all experiment density of 1.41 found by Pomeroy and Mitton (1951) for dry cal lude. The axially projected differences in density, which are important in causing meridional small-angle X-ray diffraction, are, however, large because of the diametral variations remaining after removal of water. Possible reasons for the diametral variations follow.

During drying, normal relaxed specimens show no appreciable change in the meridional wide-angle 2.86 Å spacing (Bear, 1944) which is now known to correspond to the axial projection per residue along single polypeptide chains, throughout the molecule in moist cases or along interband portions largely in dry instances. The most direct reason for assuming that the wide-angle diffraction of dry fibrils is produced largely by interbands, and is related little to bands, comes from the fact that electron stains and tanning agents have relatively little effect on wide-angle diffraction (Bolduan, Salo and Bear, 1951) Drying, staining and tanning do affect small-angle diffraction; for example, dehydration decreased the macroperiod from 660 Å. (moist) to 603 Å. (dry) in the kangaroo tail tendon studied by Rougvie and Bear (1953).

On the assumption that this axial contraction occurs chiefly at bands, one can show that as much as about 20 per cent variation in diameter between band and interband may be required if their

intrinsic densities remain nearly equal. This would correspond to electron density projection differences of around 40 per cent. Defects or overlaps because of molecular ends would occur at about one out of four molecules across an involved cross-section, to produce roughly 25 per cent variations in projected density or half that in diametral differences. Experimental fluctuations in diameter as high as 15 per cent have been reported (Nutting and Borasky, 1948).

In summary, there are two possible sources for the projected

ably of lesser magnitude than the sizeable ones encountered in the corrugated dry fibril. Absolute measures of densities and diameters would be useful in further interpretation of these factors.

THE SIGNIFICANCE OF THE 'BACKGROUNDS'

The backgrounds which were found necessary additions to the

Of the two differences, we believe the former to be best established, the latter is more sensitive to assumptions involved in derivation of the moist fibril models.

The length of 0.54 for background of moist fibrils agrees with indications from Patterson plots, as described above. In the dry material, however, Tomlin and Worthington (1956) employed a

result more in line with electron-optical data. Note that Fig. 21 of Nemetschek, Grassmann and Hoffmann (1955), which summarizes their results in terms of band position measurements, shows a lack of bands between d and e, which region also corresponds to the minimum in our curve for density of dry material.

The actual background length used for the dry fibril may be somewhat arbitrary, dependent on the representation used for bands. Since the long background occurs over the region where bands are most concentrated, its addition to the model in effect corrects for inadequacies of detail in the assumed bands.

Tomlin and Worthington added further details to their dry model which, in spite of incorrect assumption of a plane of symmetry,

same way, gave a better residual of 0.20. Similar comparisons between moist fibril models yielded residuals of 0.21 for theirs, 0.18 for ours, an expression essentially of improvement secured by adding electron-optical information about bands. Our procedures did not include extensive attempts to minimize residuals as such but did include optical canvassing of structures as described above.

We believe that it is important to consider the finer band details, so that backgrounds may be selected with greater accuracy relative to both length and location. When this is done, the difference between both characteristics for backgrounds of moist and dry fibrils reveals their essentially different meanings, which otherwise would be overlooked.

The background of the dry fibril may be said to disappear when water is added, causing instead appearance of a new background which may be at a very different place. Cohen (1954) found that tension alone causes dry kangaroo tendon (at a macroperiod of 700 Å) to yield a pattern resembling that of moist samples, showing the alternation of line intensities which requires the new background. Since both hydration and tension may be expected to straighten ... distortions at bands, the interpre-

structure to X-ray small-angle diffraction.

The meaning of the background in the moist fibril is more difficult to determine. There are perhaps three chief possibilities: (1) certain bands, d, e, a_2 and a_1, may, even when moist, retain more ... which slightly enlarges ... n greater ... the axis; (2) these same bands may contain intrinsically more electron-dense residues than do b_1, b_2 and c; and (3) the background may be

related to the locations where molecular ends overlap ('interstitial' segments of Tomlin), or the $b_1 - b_1 - c$ trough may represent places where molecular ends are pulled away from contact ('defects' of Tomlin).

One cannot clearly decide between these causes at the moment, and indeed more than one may be operative. Relative to (2) above [illegible]

with optical methods which simulated an experiment of Bolduan, Salo and Bear (1951). These authors found that that calgon staining of untreated or deaminated kangaroo tendon gave preparations in which, respectively, chiefly arginine plus lysine or arginine alone were tagged, with accompanying changes in small-angle line intensities.

CONCLUSION

In approaching the problem of electron density distribution from small-angle X-ray diffraction and electron-optical information, one naturally expects that the elimination of water will facilitate examination of the protein component alone. The results of the two methods applied to dry samples can be said thus far to have provided maps of material distribution expressed as band and interband locations or plots of electron density along the typical fibril. These remain, however, rather non-specific with respect to interpretations in terms of residue or molecule locations, which can only be very roughly surmised by indirect arguments of the type given in this paper.

One of the chief disadvantages of normal, relaxed dry material turns out to be the diffusion of structure overlaying the basic band and interband distribution, expressed by the distortional background covering much of each macroperiod. When this is removed by [illegible]

quences, are distributed along the fibril.

Particular interest attaches to the meaning of the shorter background herein indicated in moist (or extended dry) fibrils to be located differently from the distortional one of relaxed dry fibrils.

Tomlin and Worthington added further details to their dry mod which, in spite of incorrect assumption of a plane of symmetry yielded results whose agreement with experimental X-ray intensitie for kangaroo tendon is expressed by a residual of 0.22. Our model, with correct symmetry, gave calculated intensities which, when scaled to and compared with their reported X-ray intensities in the same way, gave a better residual of 0.20. Similar comparisons between moist fibril models yielded residuals of 0.21 for theirs, 0.18 for ours, an expression essentially of improvement secured by adding electron-optical information about bands. Our procedures did not include extensive attempts to minimize residuals as such but did include optical canvassing of structures as described above.

We believe that it is important to consider the finer band details, so that backgrounds may be selected with greater accuracy relative to both length and location. When this is done, the difference between both characteristics for backgrounds of moist and dry fibrils reveals their essentially different meanings, which otherwise would be overlooked

The background of the dry fibril may be said to disappear when water is added, causing instead appearance of a new background which may be at a very different place. Cohen (1954) found that tension alone causes dry kangaroo tendon (at a macroperiod of 700 Å) to yield a pattern resembling that of moist samples, showing the alternation of line intensities which requires the new background. Since both hydration and tension may be expected to straighten collagen molecules, thus removing distortions at bands, the interpretation of the above facts would be that the background of dry fibrils is merely a diffused base to the bands resulting from distortions produced by dehydration. It is then apparent that this distortional background may be similar to the modulating function which Burge and Randall (1955) found it necessary to introduce in relating band structure to X-ray small-angle diffraction.

The meaning of the background in the moist fibril is more difficult to determine. There are perhaps three chief possibilities: (1) certain bands, d, e, a_1 and a_2, may, even when moist, retain more persistently than others a degree of distortion which slightly enlarges the fibrillar diameter in their neighbourhood to result in greater material content and electron density projection on the axis; (2) these same bands may contain intrinsically more electron-dense residues than do b_1, b_2 and c; and (3) the background may be

related to the locations where molecular ends overlap ('interstitial' segments of Tomlin), or the $b_1 - b_2 - c$ trough may represent places where molecular ends are pulled away from contact ('defects' of Tomlin).

One cannot clearly decide between these causes at the moment, and indeed more than one may be operative. Relative to (2) above :have some preliminary evidence that partitioning of arginine and sine between $d - a_1$, and $b_1 - c$ locations, respectively, could be at ast a partial cause of their density differences. This work was done vith optical methods which simulated an experiment of Bolduan, ialo and Bear (1951). These authors found that that calgon staining of untreated or deaminated kangaroo tendon gave preparations in which, respectively, chiefly arginine plus lysine or arginine alone were tagged, with accompanying changes in small-angle line intensities.

CONCLUSION

In approaching the problem of electron density distribution from small-angle X-ray diffraction and electron-optical information, one naturally expects that the elimination of water will facilitate examination of the protein component alone. The results of the two methods applied to dry samples can be said thus far to have provided maps of material distribution expressed as band and interband locations or plots of electron density along the typical fibril. These remain, however, rather non-specific with respect to interpretations in terms of residue or molecule locations, which can only be very roughly surmised by indirect arguments of the type given in thi paper.

One of the chief disadvantages of normal, relaxed dry materi turns out to be the diffusion of structure overlaying the basic bar and interband distribution, expressed by the distortional backgrou covering much of each macroperiod. When this is removed hydration (or tension) one then has remaining smaller density var tions, but ones, nevertheless, which may be more significant determination of how collagen molecules, with their residue quences, are distributed along the fibril.

Particular interest attaches to the meaning of the shorter b ground herein indicated in moist (or extended dry) fibrils t located differently from the distortional one of relaxed dry f

This short background is largely responsible for the marked alte tion of small-angle line intensities characteristic of moist specim of collagen from vertebrate animals. It has been observed even w *Thyone* collagen (an echinoderm, 'sea cucumber', studied by Mar Bear and Blake, 1949), whose pattern after dehydration is, neverth less, significantly different from the commoner collagens with respec to intensity distribution. Thus, the intensity alternation, related to the shorter background, probably reflects a rather fundamental feature of the fibrillar structure of a wide variety of collagens. The importance of this phenomenon for general fibrillar structure of collagens makes it worthy of further detailed study.

In this paper we have given theoretical and experimental evidence supporting the following views regarding the distribution of matter along the collagen fibril:

(1) Axial electron density projections, bridging electron-optical and meridional small-angle X-ray diffraction data, are presented for moist and dry kangaroo tendon fibrils. In addition to properly located major band densities, one must add 'backgrounds' which cover different fractions of the macroperiod and may have different axial locations in the moist and dry cases.

(2) In the dry fibril background represents a distortional diffusion of structure at the concentrated band region underlying bands from *e* through *a*, *b* and *c* to *d*. Moisture removes this background as the molecules become straightened.

(3) With distortional background removed, a shorter background apparently covering bands *d*, *e*, a_1 and a_2 is disclosed. This background is of uncertain significance, although reasons are cited leading to the belief that it represents a fundamental feature of fibril structure, found widely in collagens.

GROUP DISCUSSION

DR GRASSMANN was pleased to hear that the chemical evidence and Dr. Bear's results were essentially in agreement. He asked if the background Dr. Bear had described might be due to amorphous matter. He also commented on the fact that even in the dry state the position of the bands is constant but the intensities are not.

DR. BEAR restated the discussion in the text of his paper relative to the

possible differences in the meanings of the backgrounds in moist and dry fibrils. In the sense that amorphous matter is distorted matter, a general distortional spreading of material about bands is one possible explanation of the backgrounds, invoked as most likely for the dry fibril. Chemical explanations seem more probable for the moist fibril. Relatively minor features of band structure have not been considered, but in a sense the assumption of background partially compensates for this.

DR. FITTON JACKSON said that Burge (1956) at King's College had

DR. NEUBERGER asked if glutamine and asparagine have electron densities similar to lysine and arginine, and if this might account for discrepancies

CHAIRMAN'S SUMMING-UP

R. E. TUNBRIDGE

In concluding this symposium it would be not only impossible but presumptive for a chairman even to attempt to summarize our deliberations. The communications, and the approved summaries of the discussions, are to be published, but, however excellent the final report, and I am sure that it will make good reading, it will miss something of the atmosphere of the symposium which only those who have been present have experienced.

Definitions are always difficult, even when one is dealing with a single language. It is not surprising therefore, that, in a rapidly [illegible]

for example has been variously interpreted as citrate soluble collagen, acid soluble collagen, or acid soluble collagen in buffered solution. It is unfortunate that in our attempts to simplify the nomenclature of the different preparations of collagen we were unable to reach complete agreement and that no agreed recommendation can be put forward by the group. Nevertheless, a considerable measure of understanding and of agreement has been reached which must inevitably be of value. Agreement was more easily reached concerning elastic tissue. This may have been due in part to the more limited interest as yet of this aspect of the connective tissue problem.

Professor Astbury, in his opening remarks, drew attention to a number of points which have been to the fore in all our discussion: the increasing importance of chemical analysis and the fact that the chemistry of collagen has temporarily at any rate, passed from the physical chemist to the analytical chemist; the difficulty in obtaining pure extracts and the need to account for the small percentage of [illegible] ...sues as well as [illegible] existence of a [illegible] yet prove to [illegible]

Comparative studies of animal tissues have indicated the variety

of collagen fibrils and possibly of collagens. The variation in th proportions of neutral and insoluble collagen in different tissues wit age and at the different stages of response to carrageenin, are merel further proof of the existence of a variety of collagens. The chemica analyses of Bowes and her colleagues, and of Grassmann for collagen, and of Partridge for elastin, and the work of Grassmann on the amino-acid sequences, are brilliant achievements but they have not answered the problem as to what significance should be attached to the finding of polysaccharide and the anomalous protein fraction. There is still no agreement whether these are impurities, due to technical imperfections, and Neuberger stressed the importance of allowing for the presence of plasma proteins in the analysis of dermis. Much evidence has been brought forward indicating the possible role of mucopolysaccharides in fibril formation both intra- and extra-cellularly. Fitton Jackson's excellent illustrations have demonstrated the possibility of intracellular fibril formation, and Schwarz and Grassmann have revealed the possible intricacies of even the collagen fibrils as a result of silver staining techniques applied to treated material studied with the electron microscope.

Reference has constantly been made to the need for caution in employing similar terms for phenomena occurring at different levels of magnitude, molecular, macro-molecular, fibril and fibre. Further, the danger of relying upon a single method of estimation and the need to check and recheck different methods were repeatedly emphasized.

The complexities of the structure, composition and function of collagenous and allied fibrils remains all too apparent, but a much greater understanding of one another's difficulties and objectives has resulted from this symposium. 'In understanding is knowledge and through knowledge cometh truth.'

Finally I should like to express on behalf of C.I.O.M.S. appreciation of your ready acceptance of the invitation to be present and to take part in this symposium. I should also like to express on your behalf our thanks to Dr. Delafresnaye and to Mrs. Tausig for their constant attention to our wants, for their unfailing kindness and consideration, and for the very efficient organization of this Symposium.

BIBLIOGRAPHY

ADAIR, G. S., DAVIS, H F and PARTRIDGE, S. M. (1951) A soluble protein derived from elastin. *Nature (Lond), 167, 605.*

ALBURN, H. E and WILLIAMS, E. C. (1950) Preparation of hyaluronic acid from various animal sources. *Ann. N.Y. Acad. Sci.*, **52**, *971.*

AMADORI, M. (1925) Prodotti di condensazione tra glucosio e p-fenetidina. *Atti Accad. Lincei*, **2**, 337.

ANDERSON, H. C and McCARTY, M. (1951) The occurrence in the rabbit of an acute phase protein analogous to human C-reactive-protein. *J. exp. Med.*, **93**, 25.

ASBOE-HANSEN, G. (1950) The origin of synovial mucin; Ehrlich's mast cell – a secretory element of the connective tissue. *Ann. rheum. Dis*, **9**, 149.

ASBOE-HANSEN, G (1953) Autoradiography of mast cells in experimental skin tumours of mice injected with radioactive sulfur (S^{35}). *Cancer Res.*, **13**, 587.

ASTBURY, W. T. (1938) Protein structure from the viewpoint of X-ray analysis. *C.R. Lab Carlsberg*, **22**, 45.

ASTBURY, W T. (1938) X-ray adventures among the proteins. *Trans. Faraday Soc.*, **34**, 377

ASTBURY, W. T. (1940) The molecular structure of the fibres of the collagen group. *J int. Soc. Leath Chem.*, **24**, 69.

ASTBURY, W. T (1945) The forms of biological molecules. In *Essays, on Growth and Form presented to D'Arcy Wentworth Thompson*, p. 309. Oxford, Clarendon Press.

ASTBURY, W. T. (1954) Personal communication.

ASTBURY, W. T. and SISSON, A. W. (1935) X-ray studies of hair, wool and related fibres *Proc. roy. Soc. A*, **150**, 533.

BADIN, J. and SCHUBERT, M (1955) Conditions of formation of euglobulin-like precipitates from serum proteins and chondroitin sulfate. *J. clin. Invest.*, **34**, 1312.

BAHR, G. F. (1950) The reconstitution of collagen fibrils as revealed by electron microscopy. *Exp. Cell Res.*, **1**, 603.

BAHR, G F. (1951) Die Feinstruktur elastischer Fasern. *Z. Anat Entw-Gesch.*, **166**, 134.

BALÓ, J and BANGA, I. (1949) Die Zerstörung der elastischen Fasern der Gefässwand. *Schweiz. Z. Path. Bakt.*, **12**, 350.

BALÓ, J and BANGA, I. (1950) Elastolytic activity of pancreatic extracts. *Biochem J.*, **46**, 384.

BALÓ, J., BANGA, I. and SCHULER, D. (1954) Vergleichende Unt chungen uber die Elastolyse der Gefässwand und des Lig Nuch histologische Schnitten. *Acta morph Acad. Sci. hung*, **14**, 141.

BANFIELD, W. G (1952) The solubility and swelling of collagen in di acid with age variations in man. *Anat. Rec.*, **114**, 157

BANFIELD, W. G. (1954) Aging of connective tissue. In *Connective Tis in Health and Disease*. Edited by G. Asboe-Hansen, p 151. Cope hagen, Munksgaard.

BANFIELD, W. G. (1955) Width and length of collagen fibrils during t development of human skin, in granulation tissue and in the skin c adult animals. *J. Geront.*, **10**, 13.

BANGA, I. (1953) Thermal contraction of collagen and its dissolution with elastase. *Nature (Lond.)*, **172**, 1099

BANGA, I. (1955) Serummucoid as a substrate of a new enzyme 'pancreatic mucase'. *3rd Int. Congr. Biochem.*, *Sect 4*, p 8

BANGA, I and BALÓ, J. (1953) Elastin and elastase. *Nature (Lond)*, **171**, 44

BANGA, I. and BALÓ, J. (1954) Studies of the elastolysis of elastin and collagen. *Acta physiol. hung.*, **6**, 235

BANGA, I. and BALÓ, J. (1956) Elastomucoproteinase and collagenmucoproteinase, the mucolytic enzymes of the pancreas *Nature (Lond.)*, **178**, 310.

BANGA, I, BALÓ, J. and SZABÓ, D. (1954) Contraction and relaxation of collagen *Nature (Lond)*, **174**, 788

BANGA, I, BALÓ, J. and SZABÓ, D (1955) *3rd Int Congr Biochem Communications*, p. 117.

BANGA, I., BALÓ, J and SZABÓ, D (1956) Metacollagen as the 'apparent' elastin. *J. Geront.*, **11**, 242.

BANGA, I, BALÓ, J and SZABÓ, D. (1956) The procollagen, as a component of collagen fibres *Acta physiol hung*, **9**, 61

BANGA, I. and SCHULER, D. (1953) Contributions to the structure of elastin with special reference to the action of elastase *Acta physiol hung.*, **4**, 13.

BANGLE, R and ALFORD, W C. (1954) The chemical basis of the periodic acid Schiff reaction of collagen fibres with reference to periodate consumption by collagen and by insulin *J Histochem Cytochem*, **2**, 62

BAZIN, S, DELAUNAY, A. and HENON, M (1956) Etudes sur le collagène VI. De l'action exercée par des endotoxines et des polyosides bactériens sur des solutions de collagène A *Ann. Inst. Pasteur*, **91**, 50

BEAR, R. S. (1944) X-ray diffraction studies on protein fibers I. The large fiber-axis period of collagen *J. Amer. chem Soc*, **66**, 1297.

BEAR, R. S. (1952) The structure of collagen fibrils In *Advances in Protein Chemistry*, **7**, 69 New York, Academic Press Inc

DEMPSEY, E. W. and LANSING, A. I. (1954) Elastic tissue. *Int. Rev. Cytol.*, **3**, 437.

DENNELL, R and MALEK, S R. A. (1956) The cuticle of the cockroach *Periplaneta americana* *Proc roy. Soc. B*, **144**, 545.

DETTMER, N. (1955) Entwicklung und Probleme elektronenmikroskopischer Bindegewebsforschung. *Arztl. Wschr.*, **10**, 708.

DETTMER, N (1956) Elektronenmikroskopische Untersuchungen am elastischen Fasersystem des Ligamentum nuchae (In the Press.)

DETTMER, N, NECKEL, I and RUSKA, H. (1951) Elektronenmikroskopische Befunde von versilberten kollagenen Fibrillen *Z. wiss. Mikr.*, **60**, 290

DETTMER, N. and SCHWARZ, W (1952-53) Elektronenmikroskopische Untersuchungen an der Interzellularsubstanz des menschlichen Sehnengewebes *Z. wiss Mikr*, **61**, 423

DETTMER, N and SCHWARZ, W. (1954) Die qualitative elektronenmikroskopische Darstellung von Stoffen mit der Gruppe CHOH-CHOH. Ein Beitrag zur Elektronenfarbung. *Z wiss. Mikr*, **61**, 423.

DISCHE, Z (1947) A specific color reaction for glucuronic acid. *J. biol Chem*, **171**, 725.

DORFMAN, A. and OTT, M. L. (1948) A turbidimetric method for the assay of hyaluronidase [illegible]

DRES [illegible]

DREYWOOD, R. (1946) Qualitative test for carbohydrate material *Industr. Engng. Chem (Anal.)*, **18**, 499

DUNPHY, J. E. and UDUPA, K N. (1955) Chemical and histochemical sequences in the normal healing of wounds. *New Engl. J. Med*, **253**, 847

EASTOE, J E and EASTOE, B (1954) The organic constituents of mammalian compact bone *Biochem J.*, **57**, 453.

EINBINDER, J and SCHUBERT, M (1950) Separation of chondroitin sulfate from cartilage. *J biol. Chem*, **185**, 725.

ELSON, L A. and MORGAN, W. T J (1933) A colorimetric method for the determination of glucosamine and chondrosamine *Biochem. J*, **27**, 1824

ENGSTROM, A and ZETTERSTROM, R (1951) Studies on the ultrastructure of bone *Exp. Cell Res*, **2**, 268.

EWALD, A (1909) Beitrage zur Kenntnis des Kollagens *Hoppe-Seyl. Z. physiol Chem*, **105**, 115.

FABER, M (1949) The human aorta. Sulfate-containing polyuronides and the deposition of cholesterol. *Arch Path (Chicago)*, **48**, 342

FAURÉ-FRÉMIET, E. (1937) La transformation thermoélastique de l'elastoidine. *J. Chim. phys*, **34**, 125.

FAURÉ-FRÉMIET, E. and WOELFFLIN, R. (1936) La température de transformation de l'elastoidine. *J Chim phys*, **33**, 801

FAWCETT, D W. (1954) Cytological and pharmacological observations on the release of histamine by mast cells *J exp Med*, **100**, 217

FISCHER, F. G. and DÖRFEL, H (1955) Die Polyuronsauren der Braunalgen (Kohlenhydrate der Algen I) *Z phys Chem*, **302**, 186.

FISHMAN, W. H, SMITH, M, THOMPSON, D B, BONNER, C. D, KASDON, S. C. and HOMBURGER, F. (1951) Investigation of glucuronic acid metabolism in human subjects *J clin. Invest*, **30**, 685

FLODIN, P. and PORATH, J. (1954) Zone electrophoresis in starch columns. *Biochem. biophys Acta*, **13**, 175

FRIBERG, U., GRAF, W. and ABERG, B. (1951) On the histochemistry of the mast cells. *Acta path. microbiol scand*, **29**, 197.

FRIEDMANN, R (1949) Characterization of sugar components of proteins *Biochem. J*, **44**, 117

FURTH, O, BRUNO, T, BOYER, R and PESCHEK, K (1937) Zur Kenntnis der Chondroitinschwefelsaure I *Biochem. Z*, **294**, 153

GALLOP, P. M (1955a) Particle size and shape in a citrate extract of ichtiocol *Arch Biochem Biophys*, **54**, 486

GALLOP, P. M (1955b) Studies on a parent gelatin from ichthyocol *Arch. Biochem. Biophys*, **54**, 501.

GARDELL, S. (1952) Some contributions to the technique of analysing polysaccharides with particular reference to animal mucopolysaccharides. *Ark. Kemi*, **4**, 449

GARDELL, S, GORDON, A. H. and AQVIST, S (1950) Electrophoresis of mucopolysaccharides in a slab of hyflo super-cel *Acta chem. scand*, **4**, 907

GARRETT, R R and FLORY, P J (1956) Evidence for a reversible first order phase transition in collagen-diluent mixtures *Nature (Lond)*, **177**, 176.

GILLMAN, T, HATHORN, M and PENN, L. (1956) Modification by sulphated hyaluronic acid of vascular and other lesions following toxic doses of calciferol (In the Press)

GILLMAN, T and PENN, J. (1956a) Studies on the repair of cutaneous wounds I. Healing of incised wounds, with reference to epidermal reactions to sutures, and the pathogenesis of carcinoma in scars *Med Proc (Suppl.)*, **2**, 121.

GILLMAN, T and PENN, J (1956b) Studies on the repair of cutaneous wounds II The healing of wounds involving loss of the superficial portion of the skin. *Med. Proc (Suppl)*, **2**, 150

GILLMAN, T., PENN, J, BRONKS, D and ROUX, M. (1953) Reactions of healing wounds and granulation tissue in man to auto-Thiersch, autodermal and homodermal grafts *Brit J plast Surg*, **6**, 153.

GILLMAN, T., PENN, J, BRONKS, D. and ROUX, M (1954) *Staining reactions of elastic fibres with reference to 'elastotic degenerations' in the human skin. Nature (Lond)*, **174**, 789.

GILLMAN, T., PENN, J, BRONKS, D. and ROUX, M. (1955a) Abnormal elastic fibres. Appearance in cutaneous carcinoma, irradiation injuries, and arterial and other degenerative connective tissue lesions in man. *Arch. Path (Chicago)*, **59**, 733.

GILLMAN, T., PENN, J, BRONKS, D. and ROUX, M. (1955b) *Possible* [illegible]

G [illegible]

GITLIN, D, LANDING, B. H and WHIPPLE, A. (1953) The localization of homologous plasma proteins in the tissue of young human beings as demonstrated with fluorescent antibodies *J. exp. Med.*, **97**, 163.

GLEGG, R. E., EIDINGER, D. and LEBLOND, C. P. (1953) Some carbohydrate components of reticular fibres. *Science*, **118**, 614.

GLICK, D. (1949) *Techniques of Histo- and Cyto-Chemistry*, p. 46 New York, Interscience Publishers, Inc.

GÖMÖRI, G (1937) Silver impregnation of reticulum in paraffin sections. *Amer J. Path.*, **13**, 993

GORTNER, A W. (1954) Effect of age on enzymic release of amino acids from aorta tissue. *J. Geront.*, **9**, 251

GOTTSCHALK, A (1951) N-substituted isoglucosamine released from mucoproteins by the influenza virus enzyme. *Nature (Lond)*, **167**, 845.

GOTTSCHALK, A. (1952) Some biochemically relevant properties of N-substituted fructosamines derived from amino acids and N-arylglucosylamines. *Biochem J.*, **52**, 455.

GOTTSCHALK, A. and LIND, P. E. (1949) Product of interaction between influenza virus enzyme and ovomucin. *Nature (Lond)*, **164**, 232.

GOTTSCHLICH, E. (1893) Ueber den Einfluss der Wärme auf Länge und Dehnbarkeit des elastischen Gewebes und des quergestreiften Muskels. *Pflugers Arch. ges. Physiol*, **54**, 109.

GRAHAM, H T., LOWRY, O H, WHEELWRIGHT, F., LENZ, M. A. and PARISH, H H., JNR. (1955) Distribution of histamine among leukocytes and platelets. *Blood*, **10**, 467.

GRASSMANN, W. (1955) Unsere heutige Kenntnis des Kollagens. *Leder*, **6**, 241.

GRASSMANN, W., ENDRES, H. and STEBER, A. (1954) Esterbindungen im Prokollagen *Z. Naturf.*, **9b**, 513.

GRASSMANN, W. and HANNIG, K. (1953) Trennung von Stoffgemischen

auf Filtrierpapier durch Ablenkung im elektrischen Feld *Hoppe-* [illegible]

GRA[illegible] ...e zur ... von ... und Chromatographie. *Hoppe-Seyl. Z. physiol. Chem.*, **299**, 258.

GRASSMANN, W., HOFMANN, U. and NEMETSCHEK, T. (1952) Die Querstreifung von Kollagenfibrillen. *Naturwissenschaften*, **39**, 215

GRASSMANN, W., HÖRMANN, H. and ENDRES, H. (1953) Eine Verbesserung der Bestimmung von Aminosäuren am Carboxylende von Peptiden durch Reduktion der Carboxylgruppe. *Chem. Ber.*, **86**, 1477.

GRASSMANN, W., HÖRMANN, H. and ENDRES, H. (1954) Säulenchromatographische Trennung von Dinitrophenylaminoalkoholen zum Zwecke der Bestimmung carboxylendständiger Aminosäuren in Peptiden und Proteinen. *Hoppe-Seyl. Z. physiol. Chem.*, **299**, 258

GRASSMANN, W., HÖRMANN, H. and ENDRES, H. (1955) Untersuchungen über die Anwendbarkeit der Reduktionsmethode zur Bestimmung carboxylendständiger Aminosäuren in Peptiden und Proteinen. *Chem. Ber.*, **88**, 102.

GRASSMANN, W., HÖRMANN, H. and HAFTER, R. (1957) Eine quantitative Bestimmung von Kohlenhydraten als Osazone Anwendung der Methode auf Kollagen und Prokollagen. *Hoppe-Seyl. Z. physiol. Chem.* (In the Press.)

GRA[illegible]

GRA[illegible]

GRA[illegible]

GRASSMANN, W. and SCHLEICH, H. (1935) Über den Kohlenhydratgehalt des Kollagens. II. Mitteilung zur Kenntnis des Kollagens *Biochem. Z.*, **277**, 230.

GREEN, R. W., ANG, K. P. and LAM, L. C. (1953) Acetylation of collagen *Biochem. J.*, **54**, 181.

GROSS, J. (1949) Structure of elastic tissue as studied with the electron microscope. *J. exp. Med.*, **89**, 699

GROSS, J. (1949/50) A study of certain connective tissue constituents with the electron microscope. *Ann. N.Y. Acad. Sci.*, **52**, 964

GROSS, J. (1950) Aging changes in the collagenous connective tissue of rat skin. A study with the electron microscope. *J. nat. Cancer Inst.*, **10**, 1353.

GROSS, J (1950) A study of the aging of collagenous connective tissue of rat skin with the electron microscope. *Amer J. Path*, **26**, 708.

GROSS, J. (1952) Aging of connective tissues. *J. Geront*, **7**, 584

GROSS, J. (1956) Properties and gelation of collagen dissolved in neutral salt solutions. *Fed Proc*, **15**, 82.

GROSS, J., HIGHBERGER, J H. and SCHMITT, F. O. (1952) Some factors involved in the fibrogenesis of collagen *in vitro*. *Proc. Soc exp. Biol (N Y)*, **80**, 462

GROSS, J, HIGHBERGER, J H. and SCHMITT, F. O. (1954) Collagen structures considered as states of aggregation of a kinetic unit. The tropocollagen particle. *Proc. nat. Acad Sci. (Wash.)*, **40**, 679

GROSS, J, HIGHBERGER, J H. and SCHMITT, F. O. (1955) Extraction of collagen from connective tissue by neutral salt solutions. *Proc. nat. Acad. Sci. (Wash.)*, **41**, 1

GROSS, J. and SCHMITT, F O. (1948) The structure of human skin collagen as studied with the electron microscope *J. exp. Med*, **88**, 555.

GROSS, J., SCHMITT, F. O and HIGHBERGER, J. H. (1952) *In vitro* fibrogenesis of collagen *Trans. 4th Josiah Macy Conf. Metabolic Interrelations*, **4**, 32.

GROSSFELD, H. (1954) Metachromasia in the living cell *Proc. Soc exp. Biol. (N Y.)*, **86**, 81.

GUSTAVSON, K. H. (1924) A new method for determination of the complex formation in chromium salts. *J. Amer. Leath. Chem. Ass*, **19**, 446

GUSTAVSON, K. H. (1926a) Specific ion effects in the behaviour of tanning agents toward collagen treated with neutral salts. *Colloid Symposium Monograph*, **4**, 79.

GUSTAVSON, K. H. (1926b) The sulfato-hydroxo-chromium compound. *J Amer Leath Chem. Ass*, **21**, 559

GUSTAVSON, K. H (1927a) The acidity of chrome leather. *J. Amer Leath Chem. Ass*, **22**, 60

GUSTAVSON, K H (1927b) The neutral salt effect in chrome tanning. *Industr. Engng Chem*, **19**, 1015.

GUSTAVSON, K H (1931) A contribution to the problem of the function of acido groups in chrome leather. *J. Amer. Leath. Chem. Ass*, **26**, 635.

GUSTAVSON, K. H. (1942a) Untersuchungen uber das Wesen der Eiweissdenaturierung durch Prufung der Reaktionsfahigkeit einiger nativer [illegible] Proteine *Biochem Z.*, **311**, 347.

G [illegible] e of the organization
Tidskr, **54**, 74

GUSTAVSON, K. H (1942c) Behaviour of collagen in combination with tanning agents towards trypsin. *Svensk. kem. Tidskr.*, **54**, 249

GUSTAVSON, K. H. (1944) Studies of chromium complexes by ion exchange. *Svensk kem. Tidskr.*, **56**, 14 (1946) **58**, 274.

GUSTAVSON, K. H (1946a) Evidence for the rupture of intermolecularly co-ordinated peptide bonds in the heat denaturation of collagen. *J. Amer Leath. Chem Ass*, **41**, 47.

GUSTAVSON, K. H (1946b) Investigation of the formation of chromium salt complexes by means of organolites *J. Colloid Sci.*, **1**, 397

GUSTAVSON, K. H. (1947a) The effect of hydrothermal denaturation of collagen upon its reactive groups. *Acta chem scand*, **1**, 581.

GUSTAVSON, K. H. (1947b) Methylglyoxal as a tanning agent. *Svensk. kem. Tidskr.*, **59**, 159

GUSTAVSON, K. H (1948) The structural stability of formaldehyde tanned collagen fibres. *J Amer Leath Chem Ass.*, **43**, 741.

GUST[illegible]

GUSTAVSON, K. H (1950a) The effect of anionic detergents on collagens of mammals and teleostei. *Acta chem. scand*, **4**, 1171

GUSTAVSON, K H. (1950b) Some contrasting effects of anionic agents on collagens of mammals and fishes *J. Amer. Leath. Chem. Ass*, **45**, 789.

GUSTAVSON, K. H (1952a) The effect of esterification of the carboxyl groups of collagen upon its combination with chromium compounds *J Amer. chem. Soc*, **74**, 4608

GUSTAVSON, K H (1952b) Some reactions of esterified collagen. *Acta chem. scand*, **6**, 1443

GUSTAVSON, K H (1953) Hydroxyproline content and degree of interchain linking of collagens from mammalian and teleost skins. *Svensk kem Tidskr*, **65**, 70

GUSTAVSON, K. H (1954a) Hydroxyproline and stability of collagen. *Acta chem scand*, **8**, 1298

GUSTAVSON, K H (1954b) The presence of interchain cross-links between hydroxy- and keto-imide groups in collagen *Acta chem. scand*, **8**, 1300

GUSTAVSON, K H (1954c) Interactions of vegetable tannins with polyamides as proof of the dominant function of the peptide bonds of [illegible]

G[illegible]

G[illegible]

G[illegible] *Nature (Lond)*, **175**, 70

GUSTAVSON, K. H. (1955b) The nature of the cross-links in collagen and gelatin. *Svensk. kem Tidskr.*, **67**, 115.

GUSTAVSON, K. H. (1955c) Evidence for the presence of the -OH... OC-link in collagen from the fixation of non-ionic chromium complexes *Acta chem. scand*, **9**, 1049.

GUSTAVSON, K. H. (1956a) *The Chemistry and Reactivity of Collagen* New York, Academic Press.

GUSTAVSON, K. H (1956b) *The Chemistry of Tanning Processes.* New York, Academic Press.

GUSTAVSON, K. H. (1956c) Approximation of the proportion of uni- and multi-point binding of basic chromium chlorides by collagen. *Ark. Kemi* (In the Press.)

HADIDIAN, Z. and PIRIE, N. W. (1948) The preparation and some properties of hyaluronic acid from human umbilical cord. *Biochem. J*, **42**, 260.

HALL, C E (1956) Visualization of individual macromolecules with the electron microscope. *Proc. nat. Acad. Sci (Wash.)*, **42**, 801

HALL, C. E., JAKUS, M. A. and SCHMITT, F. O. (1942) Electron microscope observations of collagen *J Amer chem Soc.*, **64**, 1234.

HALL, D A. (1951) Elastin from human tissue and from ox ligament. *Nature (Lond)*, **168**, 513.

HALL, D. A. (1953) Studies on the complex nature of the elastin-elastase system. *Biochem J.*, **55**, xxxvi.

HALL, D. A. (1955) The reaction between elastase and elastic tissue I. The substrate. *Biochem. J.*, **59**, 459.

HALL, D. A. (1956) The complex nature of the enzyme elastase. *Arch. Biochem Biophys.* (In the Press.)

HALL, D A. and GARDINER, J E. (1955) The reaction between elastase and elastic tissue. II Preparation and properties of the enzyme *Biochem. J*, **59**, 465.

HALL, D. A., KEECH, M. K., REED, R, SAXL, H., TUNBRIDGE, R. E. and WOOD, M J (1955) Collagen and elastin in connective tissue. *J. Geront*, **10**, 388

HALL, D. A., REED, R. and TUNBRIDGE, R. E. (1952) Structure of elastic tissue. *Nature (Lond.)*, **170**, 264.

HALL, D. A., REED, R. and TUNBRIDGE, R. E. (1955) Electron microscope studies of elastic tissue *Exp Cell Res*, **8**, 35

HAM, A W. (1932) Mechanisms of calcification in heart and aorta in hypervitaminosis D. *Arch Path. (Chicago)*, **14**, 613.

HAM, A. W. and LEWIS, M. D. (1934) Experimental intimal sclerosis of the coronary arteries of rats. *Arch. Path. (Chicago)*, **17**, 356.

HAM, A. W. and PORTUONDO, B. C. (1933) Relation of serum calcium to pathologic calcifications of hypervitaminosis D. *Arch. Path (Chicago)* **16**, 1.

HARKNESS, R. D., MARKO, A. M , MUIR, H. M. and NEUBERGER, A. (1954) The metabolism of collagen and other proteins of the skin of rabbits *Biochem. J*, 56, 558.

HED

HED

ein neues Syndrom. 64, 942.

HERINGA, G C. and KOLMEIJER, N. H. (1926) Investigations with roentgenrays of the structure of the collagenic substance *Verh. Akad. Wet. Amst.*, 29, 1092.

HERINGA, G C. and LOHR, H A. (1926) An inquiry into the physicochemical structure of the collagenic substance. I. *Verh. Akad. Wet. Amst*, 29, 1086

HERINGA, G. C. and MINNAERT, M (1926) An inquiry into the physicochemical structure of the collagenic substance II. *Verh. Akad. Wet. Amst*, 29, 1091.

HERRATH, E. v. and DETTMER, N. (1951) Elektronenmikroskopische Untersuchungen an Gitterfasern *Z wiss Mikr*, 60, 282.

HIGHBERGER, J. H (1947) The structural stability of the collagen fiber in relation to the mechanism of tanning. *J Amer Leath. Chem Ass.*, 42, 493

HIGHBERGER, J H., GROSS, J. and SCHMITT, F. O (1951) The interaction of mucoprotein with soluble collagen. An electron microscope study. *Proc nat. Acad Sci. (Wash)*, 37, 286

HOF

HOF

Querstreifung von Kollagenfibrillen und ihre Veranderung im Elektronenmikroskop *Z. Naturf*, 7b, 509

HOLMGREN, H. and WILANDER, O (1937) Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen *Z mikr.-anat. Forsch*, 42, 242.

HOMAN, J. D. H and LENS, J (1948) A simple method for the purification of heparin *Biochim biophys. Acta*, 2, 333

HOOFF, A VAN DEN (1952) The ageing of collagen fibrils in the rabbit ear *Verh Akad Wet Amst*, C55, 628.

HOOFF, A VAN DEN (1952) Electron microscopy of cornea and sclera connective tissue. *Verh Akad Wet. Amst.*, C55, 628

HORMANN, H (1956) Die Reduktion von Carbonylverbindungen durch komplexe Hydride *Angew Chem.*, 68, 601

HORMANN, H, GRASSMANN, W, WUNSCH, E and PRELLER, H. (1956)

Die Beresterung von Peptiden und ihre Bedeutung für die Bestimmung carboxylendständiger Aminosauren nach der Reduktionsmethode. *Chem. Ber*, **89**, 933.

HUGGINS, M L. (1954) Structure of collagen. *J. Amer chem Soc*, **76** 4045.

IRVING, E A and TOMLIN, S. G. (1954) Collagen, reticulum and their argyrophilic properties *Proc. roy. Soc. B*, **142**, 113.

JACKSON, D. S. (1953) Chondroitin sulphuric acid as a factor in the stability of tendon. *Biochem. J.*, **54**, 638.

JACKSON, D. S (1954) The nature of collagen chondroitin sulphate linkage in tendon *Biochem J.*, **56**, 699

JACKSON, D. S. (1956a) The stimulation of connective tissue formation by carrageenin. *Biochem J.*, **62**, 25P.

JACKSON, D. S (1956b) The formation and removal of collagen in the carrageenin granuloma. *Biochem. J.*, **64**, 8P

JACKSON, D. S (1957) This Conference.

JACKSON, D. S. and FESSLER, J (1955) Isolation and properties of a collagen soluble in salt solution at neutral pH. *Nature (Lond)*, **176**, 69

JACKSON, S. F (1954a) Fibrogenesis in connective tissues. *Nature (Lond.)*, **173**, 950.

JACKSON, S. F (1954b) The formation of connective and skeletal tissues *Proc. roy. Soc. B*, **142**, 536

JACKSON, S. F. (1955) Cytoplasmic granules in fibrogenic cells. *Nature (Lond)*, **175**, 39

JACKSON, S. F. (1956) The morphogenesis of avian tendon. *Proc. roy. Soc. B*, **144**, 566.

JACKSON, S. F. and RANDALL, J T. (1956) *Ciba Symposium. Bone Structure and Metabolism* (1955), p 47 Edited by G. E. W. Wolstenholme and C. M. O'Connor. London, Churchill.

JACKSON, S F. and SMITH, R. H. (1955) Fibrous proteins and their biological significance. *Symposia of the Society for Experimental Biology*, No. 9. Cambridge University Press.

JACOBSON, K. H. and LOLLAR, R. M. (1951) Wet tension testing studies as influenced by chemical modifications of collagen *J. Amer. Leath Chem Ass.*, **46**, 7

JAHNKE, A (1956) Elektronenmikroskopische Untersuchungen uber die Interzellularsubstanz der menschlichen Aortenklappen. *Virchows Archiv*, **329**, 486.

JAQUES, L. B (1943) The reaction of heparin with proteins and complex bases. *Biochem. J.*, **37**, 189.

JAQUES, L. B. and CHARLES, A. F. (1941) The assay of heparin. *Quart J Pharm*, **14**, 1.

JEANLOZ, R. and FORCHIELLI, E (1950) Studies on hyaluronic acid and

related substances. I Preparation of hyaluronic acid and derivatives from human umbilical cord. *J. biol. Chem*, **186**, 495

JELLEY, E. E. and PONTIUS, R. B. (1955) The diffusion of dyes in gelatin *J photogr Sci.*, **2**, 15.

JONES, H. [illegible]
beta-p [illegible]

JONES, J I. [illegible]
Nature (Lond), **165**, 685.

JORPES, J. E. (1929) Eine Methode zur Darstellung der Chondroitinschwefelsaure *Biochem Z*, **204**, 354

JORPES, J E (1935) The chemistry of heparin *Biochem. J*, **29**, 1817

JORPES, J E. (1946) *Heparin in the Treatment of Thrombosis*, p 62. London, Oxford University Press.

JORPES, J E, WERNER, B. and ABERG, B (1948) The Fuchsinsulfurous acid test after periodate oxidation of heparin and allied polysaccharides *J. biol Chem*, **176**, 277.

JULÉN, C, SNELLMAN, O. and SYLVÉN, B (1950) Cytological and fractionation studies on the cytoplasmic constituents of tissue mast cells [illegible]

[illegible]

Die Konfiguration der Glucosamin- und Chondrosaminsaure. *Helv chim acta*, **20**, 407

KEECH, M K (1954) The effect of collagenase and trypsin on collagen An electron microscope study. *Anat Rec*, **119**, 139

KING, A. L. (1946) Pressure-volume relation for cylindrical tubes with elastomeric walls the human aorta *J appl Phys*, **17**, 501

KNOX, W E, AERBACH, V H. and LIN, E C. C (1956) Enzymatic and metabolic adaptations in animals *Physiol Rev*, **36**, 164

KOKSAL, M. (1953) Extraction of a heparin-like substance from mast cell granules in mouse connective tissue *Nature (Lond)*, **172**, 733

KRAMER, H. and LITTLE, K. (1953) In *Nature and Structure of Collagen*, p 33 London, Butterworth

KRAMER, H and WINDRUM, G M (1953) Metachromasia after treating tissue sections with sulphuric acid *J clin Path*, **6**, 239

KRONER, T D, TABROFF, W and MCGARR, J J (1953) Peptides isolated from a partial hydrolysate of steer hide collagen *J. Amer chem Soc.*, **75**, 4084.

KRONER, T D, TABROFF, W and MCGARR, J J (1955) Peptides isolated from a partial hydrolysate of steer hide collagen II. Evidence for the prolyl-hydroxyproline linkage in collagen *J Amer. chem. Soc*, **77**, 3356.

KÜHN, K., HOFMANN, U. and GRASSMANN, W. (1956) Querstreifung und Perjodat-Silberurotropin-Reaktion bei verschiedenen Kollagenarten *Z. Naturf*, **11b**, 581.

KUHN, K, HOFMANN, U. and GRASSMANN, W. (1956) Die Feinstruktur verschiedener kollagener Fibrillen und ihr Verhalten bei der Silberreaktion *Z. Naturf* (In the Press.)

KUHN, W. (1956) Discussion remark. *Symposium on Experimental Research on Ageing*, April 1956 Basel. Basel, Birkhauser.

KUNKEL, H. G and TISELIUS, A (1951) Electrophoresis of proteins on filter paper. *J. gen. Physiol.*, **35**, 89

KUNTZEL, A. (1937) Über die Verleimung des Kollagens *Stiasny Festschrift*, p. 191 Edited by K. H. Gustavson Darmstadt, E Roether.

KUNTZEL, A. (1941) Die Struktur der Kollagenfaser. *Kolloidzschr.*, **96**, 273.

KÜNTZEL, A, RIESS, C, PAPAYANNIS, A. and VOGL, H. (1934) Über die Zusammensetzung der an die Hautfaser gebundenen Chromverbindungen im Chromleder *Collegium*, No. 770, 261.

KUPFFER, C (1875) Ueber Sternzellen der Leber. *Arch mikr Anat*, **12**, 353.

LANHAM, A. F. and PANKHURST, K. G. A. (1956) Surface film studies of the mechanism of vegetable tanning. *Trans. Faraday Soc.*, **52**, 521.

LANSING, A. I. (1955) Ageing of elastic tissue and the systemic effects of [illegible]

L[illegible]

[illegible] *Proc. Soc. exp. Biol. (N.Y.)* 76, 714 [illegible]

[illegible] W. (1952) [illegible] c fibers as revealed by elastase and by electron microscopy *Anat Rec.*, **114**, 555

LEHNER, J. (1924) Das Mastzellen-Problem und die Metachromasie-Frage *Ergebn Anat EntwGesch*, **25**, 67.

LERCH, H. (1950) Ueber Warmeschrumpfung des Kollagengewebes *Gegenbaurs Jb.*, **90**, 192; 206.

LERCH, H. (1953) Pathologische Wärmeschrumpfungen am Sehnengewebe. *Arch. orthop. Unfall-Chir.*, **46**, 58.

LEVENE, P. A and LA FORGE, F. B. (1914) About conjugated sulfuric acid from tendomucoid. *J. biol. Chem.*, **18**, 237.

LEWIS, W. H. (1941) Pinocytosis Drinking by cells *Anat Rec*, **80**, 395.

LILLIE, R D. (1952) Histochemistry of connective tissue. *Lab Invest*, **1**, 30.

LINDNER, E. (1954) Zur Struktur der Fasern und Fibrillen des Plasmocytoms *Ärztl Forsch.*, **8**, 487.

LINKE, K. W. (1955) Elektronenmikroskopische Untersuchung uber die Differenzierung der Interzellularsubstanz der menschlichen Lederhaut. *Z. Zellforsch.*, **42**, 331.

LINKER, A., MEYER, K. and HOFFMAN, P. (1956) The production of unsaturated uronides by bacterial hyaluronidases *J biol. Chem.*, **219**, 13.

LINKER, A., MEYER, K and WEISSMANN, B. (1955) Enzymatic formation of monosaccharides from hyaluronate. *J. biol Chem.*, **213**, 237.

LITTLE, K. and KRAMER, H. (1952) Nature of reticulin. *Nature (Lond.)*, **170**, 499

LITTLE, K. and WINDRUM, G. M. (1954) A lipid component of reticulin *Nature (Lond)*, **174**, 789

LLOYD, D. J. and GARROD, M (1946) *Fibrous Proteins*, p. 24 Bradford· Society of Dyers and Colorists.

LLOYD, D. J , MARRIOTT, R H. and PLEASS, W. B. (1933) Swelling of protein fibres. I. Swelling of collagen. *Trans. Faraday Soc* , **29**, 554

LLOYD, P. F. (1956) Connective tissue and its changes *Nature (Lond)*, **177**, 467.

LLOYD, P. F., CZERKAWSKI, J. W and HALL, D A. (1957) An improved zonal electrophoresis apparatus (In the Press)

LOEWEN, W. A. (1955a) The binding collagen-mucopolysaccharide in connective tissue. *Acta anat. (Basel)*, **24**, 217.

LOEWEN, W. A. (1955b) The nature of the complex binding between collagen and mucopolysaccharide in connective tissue. *Acta physiol. pharmacol. neerl* , **4**, 243.

LUNDGREN, H P. (1954) Some chemical experiments on wool. *Text Res. (J)*, **24**, 342.

LUNDGREN, H P. and BINKLEY, C. H. (1954) Application of rhodamine-B to interaction studies in proteins and simple model systems *J Polymer Sci* , **14**, 139.

MACCONAILL, M A. (1955) Double illumination microscopy. *Nature (Lond)*, **176**, 877.

M'EWEN, M B. and PRATT, M I. (1953) In *Nature and Structure of Collagen*, p 158. London, Butterworth.

MCFARLANE, A. S (1956) Labelling of plasma proteins with radioactive iodine. *Biochem J* , **62**, 135

MCMANUS, J F. A (1946) The histological demonstration of mucin after periodic acid. *Nature (Lond)*, **158**, 202.

MALL, F. P. (1888) Reticulated and yellow elastic tissues *Anat. Anz* , **3**, 397

MALMGREN, H and SYLVÉN, B (1952) Biophysical and physiological investigations on cartilage and other mesenchymal tissues V. Identification of the polysaccharide of bovine nuclei pulposi. *Biochim. biophys. Acta*, **9**, 706

MAO, T. J and RODDY, W. T. (1950) The dry strength of collagen fiber aggregates. *J Amer Leath Chem. Ass.*, **45**, 131.

MARBET, R. and WINTERSTEIN, A. (1951) β-Heparin, ein neuer, blutgerinnungshemmender Mucoitinschwefelsäureester *Helv. chim acta*, **34**, 2311.

MARKS, M H., BEAR, R S and BLAKE, C. H. (1949) X-ray diffraction evidence of collagen-type protein fibers in the echinodermata, coelenterata and porifera. *J exp. Zool.*, **111**, 55.

MARTIN, A. V. W. (1953) Fine structure of cartilage matrix In *Nature and Structure of Collagen*, p. 129. London, Butterworth.

MATHEWS, M B. (1953) Chondroitin-sulfuric acid, a linear polyelectrolyte *Arch. Biochem Biophys*, **43**, 181.

MEGGY, A. B. and SIKORSKI, J (1956) Dimorphism of polyglycine. *Nature (Lond)*, **177**, 326.

MEYER, K. (1945) Mucoids and glycoproteins In *Advances in Protein Chemistry*, **2**, p. 254. New York, Academic Press.

MEYER, K. (1948) Highly viscous sodium hyaluronate. *J. biol. Chem*, **176**, 993.

MEYER, K (1954) The chemistry of ground substances of connective tissue. In Asboe-Hansen, G. *Connective Tissue in Health and Disease.* Copenhagen.

MEYER, K. and CHAFFEE, E (1941) The mucopolysaccharides of skin. *J. biol. Chem.*, **138**, 491.

MEYER, K., DAVIDSON, E, LINKER, A. and HOFFMAN, P. (1956) The acid mucopolysaccharides of connective tissue. *Biochim. biophys. Acta*, **21**, 506

MEYER, K, LINKER, A., DAVIDSON, E. A. and WEISSMANN, B. (1953) The mucopolysaccharides of bovine cornea. *J. biol Chem*, **205**, 611.

MEYER, K. and RAPPORT, M M (1951) The mucopolysaccharides of the [illegible] **113**, 596.

[illegible] VI. The preparation [illegible] 07.

MEYER, K. H. and FERRI, C. (1936) Die elastischen Eigenschaften der elastischen und der kollagen Fasern und ihre moleculare Deutung. *Pflugers Arch ges. Physiol*, **238**, 78.

MEYER, K. H, ODIER, M. E. and SIEGRIST, A. E. (1948) Constitution de l'acide chondroitine-sulfurique. *Helv. chim. acta*, **31**, 1400.

MICHEEL, F. and KLEMER, A. (1951) N-Glucoside von Aminosauren. *Chem. Ber.*, **84**, 212.

MICHEEL, F and KLEMER, A. (1956) D-Glucosederivate von Proteinen. *Chem Ber.*, **89**, 1238.

MICHELS, N. A (1938) *Handbook of Hematology*, **1**, p. 231. (Edited by H Downey). New York, P. B. Hoeber Inc.

MILLER, L. L., BALE, W F, YUILE, C. L., MASTERS, R. E., TISHKOFF, G. H. and WHIPPLE, G. H. (1949) The use of radioactive lysin in studies of protein metabolism *J. exp. Med*, **90**, 297.

MONTAGNA, W., EISEN, A. Z. and GOLDMAN, A. S. (1954) The tinctorial behaviour of human mast cells. *Quart J. micr. Sci*, **95**, 1.

MOORE, S and STEIN, W. H. (1951) Chromatography of amino acid on sulphonated polystyrene resins. *J. biol. Chem*, **192**, 663.

MOSS, J A (1955) The carbohydrate of collagen. *Biochem. J*, **61**, 151.

MYANT, N. B. (1952) Observations on the metabolism of human gamma globulin labelled by radioactive iodine. *Clin. Sci*, **11**, 191.

NAGEOTTE, J. (1927) Action des sels neutres sur la formation du caillot artificiel de collagène *C R Soc. Biol., Paris*, **96**, 828.

NAUCK, E T (1931) Die Wellung der Sehnenfasern, ihre Ursache und ihre funktionelle Bedeutung *Gegenbaurs Jb*, **68**, 79.

NEMETSCHEK, T, GRASSMANN, W and HOFMANN, U. (1955) Über die hochunterteilte Querstreifung des Kollagens. *Z. Naturf*, **10b**, 61.

NEUBERGER, A, PERRONE, J. C and SLACK, H. G B. (1951) The relative metabolic inertia of tendon collagen in the rat. *Biochem J.*, **49**, 199

NEUMAN, R E. (1949) Amino acid composition of gelatins, collagens and elastins from different sources *Arch Biochem*, **24**, 289.

NEUMAN, R. E and LOGAN, M. A (1950) The determination of hydroxyproline. *J biol Chem*, **184**, 299

NEURATH, N. and SCHWERT, G W. (1950) The mode of action of the crystalline pancreatic proteolytic enzymes *Chem Rev.*, **46**, 69.

NODA, H (1955) Physico-chemical studies of the soluble collagen of rat-tail tendon *Biochim biophys Acta*, **17**, 92.

NODA, H. and WYCKOFF, R W. A. (1951) The electron microscopy of reprecipitated collagen *Biochim biophys Acta*, **7**, 494

NORTH, A C T, COWAN, P M and RANDALL, J T. (1954) Structural units in collagen fibrils *Nature (Lond)*, **174**, 1142.

NOVIKOFF, A. B., PODBER, E, RYAN, C and NOE, R. (1952) Biochemical heterogeneity of the cytoplasmic particles isolated from rat liver homogenates *Fed Proc*, **11**, 265

NUTTING, G. C and BORASKY, R (1948) Electron microscopy of collagen *J Amer. Leath Chem Ass*, **43**, 96.

OREKHOVITCH, V N (1952) *2nd Int Congr Biochem Communica*
p 106.

OREKHOVITCH, V N (1955) *3rd Int Congr Biochem Communications*, p.

OREKHOVITCH, V. N and SHPIKITER, V O (1955) Molecular weight a degree of asymmetry of procollagen *Biokhimija*, **20**, 438

OREKHOVITCH, V N, TUSTANOWSKI, A A, OREKHOVITCH, K. D. a PLOTNIKOVA, N. E (1948) The procollagen of hide. *Biokhimij*, **13**, 55.

OREKHOVITCH, V. N., STANOWSKI, A. A. and PLOTNIKOVA, N. E. (1948) Isolation of crystalline proteins of a new type (procollagen) from ... .R. Acad. Sci U.R.S.S., 60, 83

ORR, S F ... studies of some polysaccharides *Biochim. biophys Acta*, **14**, 173.

PAHLKE, G. (1954) Elektronenmikroskopische Untersuchungen an der Interzellularsubstanz des menschlichen Sehnengewebes. *Z. Zellforsch* **39**, 421.

PARTRIDGE, S. M (1948) The chemistry of connective tissue *Biochem J*, **43**, 387.

PARTRIDGE, S M. and DAVIS, H F. (1950) Preferential release of aspartic acid during the hydrolysis of proteins. *Nature* (*Lond*), **165**, 62.

PARTRIDGE, S M. and DAVIS, H. F. (1955) Composition of the soluble proteins derived from elastin *Biochem. J.*, **61**, 21.

PARTRIDGE, S. M., DAVIS, H F. and ADAIR, G. S. (1955) The chemistry of connective tissue. II Soluble proteins derived from partial hydrolysis of elastin *Biochem. J.*, **61**, 11.

PAULING, L. and COREY, R. B. (1953) Compound helical configurations of polypeptide chains· structure of proteins of the -keratin type. *Nature* (*Lond.*), **171**, 59.

PAULING, L and NIEMANN, C. (1939) The structure of proteins. *J. Amer. chem. Soc.*, **61**, 1860.

PAYSA, N. and KORN, E D (1956) Enzymatic degradation of heparin. *Fed. Proc.*, **15**, 325.

PEARCE, R H and WATSON, E. M. (1949) The mucopolysaccharides of human skin. *Canad. J Res., Section E*, **27**, 43.

PEASE, D C. (1955) Fine structures of the kidney seen by the electron microscope. *J. Histochem. Cytochem*, **3**, 295.

PENNEY, J. R and BALFOUR, B. M. (1949) The effect of vitamin C on mucopolysaccharide production in wound healing. *J. Path. Bact*, **61**, 171.

POMEROY, C. D. and MITTON, R. G. (1951) The real densities of chrome- and vegetable-tanned leathers. *J. Soc. Leath Tr. Chem*, **35**, 360.

PORATH, J. (1956) Methodological studies of zone-electrophoresis in vertical columns. I. Fractionation in cellulose powder columns of substances of low molecular weight exemplified by amino aci... related compounds. *Biochim. biophys. Acta*, **22**, 151.

PORTER, K. R. and VANAMEE, P. (1949) Observations on ...

F ...

Hyalin. *Phys. Verh., Mosbach*, **5**, 108.

RAMACHANDRAN, G N and KARTHA, G. (1954-56) Structure of collagen. *Nature (Lond.)*, **174**, 269, **176**, 593; **177**, 710

RAMACHANDRAN, G. N and KARTHA, G. (1955) Studies on collagen I. Structure of the collagen group of proteins *Proc. Ind. Acad Sci.*, **42**, 215.

RANDALL, J T. (1953) Discussion on the structure of collagen *Nature and Structure of Collagen*, p. 232 London, Butterworth.

RANDALL, J. T (1954) Observations on the collagen system. *J. Soc. Leath. Tr. Chem*, **38**, 362.

RANDALL, J. T. (1954) Observations on the collagen system. *Nature (Lond.)*, **174**, 853

RANDALL, J T. (1954) Structural units in collagen fibrils. *Nature (Lond.)*, **174**, 1142

REED, R. and RUDALL, K M (1948) Electron microscope studies on the structure of earthworm cuticles *Biochim biophys Acta*, **2**, 7

REED, R, WOOD, M J. and KEECH, M K. (1956) Helical nature of the collagen fibril. *Nature (Lond.)*, **177**, 697

REMINGTON, C. (1931) The carbohydrate complex of the serum proteins. II. Improved method for isolation and redetermination of structure Isolation of glucosamindimannose from proteins of ox blood *Biochem. J.*, **25**, 1062.

REMINGTON, J W. (1945) Pulse wave velocity and stroke volume of beat. *Amer J. Physiol.*, **144**, 536

RICH, A. and CRICK, F H. C (1955) The structure of collagen *Nature (Lond)*, **176**, 915

RILEY, J F, SHEPHERD, D. M, WEST, G B and STROUD, S. W. (1955) Function of heparin. *Nature (Lond.)*, **176**, 1123.

RILEY, J. F and WEST, G. B. (1953) The presence of histamine in tissue mast cells. *J Physiol*, **120**, 528

RILEY, J. F and WEST, G B (1955) Tissue mast cells. Studies with a histamine-liberator of low toxicity (compound 48/80). *J Path Bact.*, **69**, 269

RINEHART, J F, FARQUHAR, M G, JUNG, H. C. and ABUL-HAJ, S. K (1953) The normal glomerulus and its basic reactions in disease *Amer. J. Path*, **29**, 21

ROBBINS, W. C., WATSON, R F, PAPPAS, G. D and PORTER, K. R (1955) Some effects of anti-collagen serum on collagen formation in tissue culture: a preliminary report *J biophys biochem. Cytol*, **1**, 381.

ROBB-SMITH, A. H. T. (1937) A device to facilitate the impregnation of reticulin fibrils in paraffin sections. *J Path. Bact*, **45**, 312.

ROBB-SMITH, A. H. T (1945) Tissue changes induced by *Cl. Welchii* type A filtrate. *Lancet*, **2**, 362.

ROBB-SMITH, A. H. T. (1952) The nature of reticulin *Trans 3rd Josiah Macy Conf Connective Tissues*, p 92.

ROBB-SMITH, A H. T. (1953) The significance of collagenase. *Nature and Structure of Collagen*, p. 14 London, Butterworth

ROB[illegible]

ROBI[illegible]
in bone collagen. *Anat. Rec*, **114**, 383.

RODDY, W. T. (1952) The dry strength of collagen fiber aggregates. *J. Amer. Leath. Chem Ass*, **47**, 98.

ROLLHÄUSER, H. (1950–54) Konstitutions- und Altersunterschiede in Festigkeit kollagener Fibrillen Untersuchungen über den submikroskopischen Bau kollagener Fasern. *Gegenbaurs Jb*, **90**, 157; **92**, 1

ROLLHAUSER, H (1954) Die Doppelbrechkraft der Sehne bei gesteigerter Muskeltätigkeit. *Z. Zellforsch*, **40**, 459.

ROSE, W G. and LUNDGREN, H. P. (1953) Polymerization of beta-propiolactone in wool *Text Res (J.)*, **23**, 930.

ROUGVIE, M A and BEAR, R. S. (1953) An X-ray diffraction investigation of swelling by collagen *J. Amer. Leath Chem Ass*, **48**, 735.

ROY, C. S. (1880) The elastic properties of the arterial wall. *J. Physiol*, **3**, 125

RUDALL, K. M. (1946) The structure of epidermal protein *Fibrous Proteins (Symposium of the Society of Dyers and Colourists)*, p 15. Leeds.

RUDALL, K. M. (1956) Personal communication.

[illegible]
studies on skin utilizing C^{14}-glucose, C^{14}-acetate, and S^{35}-sodium sulfate *J biol Chem*, **218**, 139.

SCHILLER, S., MATHEWS, M. B., GOLDFARBER, L., LUDOWEIG, J. and DORFMAN, A. (1955) The metabolism of mucopolysaccharides in animals II. Studies in skin utilizing labelled acetate *J biol. Chem*, **212**, 531.

SCHILLER, S, MATHEWS, M B, JEFFERSON, H, LUDOWEIG, J. and DORFMAN, A. (1954) The metabolism of mucopolysaccharides of animals I Isolation from skin. *J biol Chem.*, **211**, 717.

SCHMID, K. (1950) Preparation and properties of an acid glyco-protein prepared from human plasma *J Amer. chem. Soc*, **72**, 2816.

SCHMID, K. (1953) Preparation and properties of serum and plasma proteins. XXIX. Separation from human plasma of polysaccharides, peptides and proteins of low molecular weight. *J. Amer. chem. Soc*, **75**, 60.

SCHMITT, F. O. and GROSS, J (1948) Further progress in the electron microscopy of collagen. *J. Amer Leath. Chem Ass.*, **43**, 658.

SCHMITT, F. O, GROSS, J and HIGHBERGER, J H. (1953) A new particle type in certain connective tissue extracts *Proc nat Acad. Sci (Wash.)*, **39**, 459.

SCHMITT, F. O, GROSS, J and HIGHBERGER, J H (1955a) Tropocollagen and the properties of fibrous collagen *Exp. Cell Res*, *Suppl 3*, 326

SCHMITT, F O, GROSS, J and HIGHBERGER, J. H (1955b) States of aggregation of collagen *Fibrous Proteins and their Biological Significance. Symposia of the Society for Experimental Biology*, **9**, 148 Cambridge University Press

SCHMITT, F. O, HALL, C. E and JAKUS, M A (1942) Electron microscopy studies on collagen *J cell comp. Physiol*, **20**, 11

SCHNEIDER, F. (1940) Über die chemische Zusammensetzung des Kollagens. *Collegium*, p 97

SCHNEIDER, F. (1949) Über die Bedeutung des Zuckers im Kollagen *Angew Chem*, **61**, 259

SCHROEDER, W. A., HONNEN, L and GREEN, F C. (1953) Chromatographic separation and identification of some peptides in partial hydrolysates of gelatin *Proc nat Acad Sci (Wash)*, **39**, 23

SCHROEDER, W. A, KAY, L M, LEGETTE, J, HONNEN, L and GREEN, F. C. (1954) The constitution of gelatin Separation and estimation of peptides in partial hydrolysates *J Amer chem Soc*, **76**, 3556.

SCHWARZ, W. (1953) Elektronenmikroskopische Untersuchungen uber den Aufbau der Sklera und der Cornea des Menschen. *Z Zellforsch*, **38**, 26

SCHWARZ, W. (1953) Elektronenmikroskopische Untersuchungen ub[er] die Differenzierung der Cornea- und Sklerafibrillen des Menscher[n] *Z Zellforsch*, **38**, 78

SCHWARZ, W. (1954) Die Zwischensubstanzen des Bindegewebes In *Kapillaren und Interstitium*, p 29 Herausgegeben von H Bartelheimer und H Kuchmeister Stuttgart, G Thieme

SCHWARZ, W (1955) Die Zwischensubstanzen des Bindegewebes *Hamburger Symposium uber Kapillaren und Interstitium* Stuttgart, G Thieme.

SCHWARZ, W (1955) Über die Morphologie der verschiedenen Bindegewebsfasern unter besonderer Berucksichtigung des Kollagens *Die Staublungenerkrankungen.* (In the Press)

SCHWARZ, W (1956) This Symposium

SCHWARZ, W and DETTMER, N (1953) Elektronenmikroskopische Untersuchung des elastischen Gewebes in der menschlichen Aorta *Virchows Archiv*, **323**, 243.

SEVAG, M G (1934) Eine neue physikalische Enteiweissungsmethode zur Darstellung biologische wirksamer Substanzen Isolierung von Kohlenhydraten aus Huhnereiweiss und Pneumococcen *Biochem. Z*, **273**, 419

SINGLETON, L. (1955) Ph.D. Thesis, Leeds University.

SLACK, H. G. B. (1956) The metabolism of sulphated polysaccharides in carrageenin-induced granuloma. *Biochem. J.*, **64**, 7P.

SMITH, E. L., KIMMEL, J. R., BROWN, D. M. and THOMPSON, E. O. P. (1955) Isolation and properties of a crystalline mercury derivative of a lysozyme from papaya latex. *J. biol. Chem.*, **215**, 67.

SMITH, H and GALLOP, R. C. (1953) The 'acid polysaccharides' of hog gastric mucosa. *Biochem. J.*, **53**, 666.

SNELLMAN, O, JENSEN, R. and SYLVÉN, B (1948) A new extraction procedure for the preparation of heparin. *Nature (Lond.)*, **161**, 639

SNELLMAN, O., JENSEN, R and SYLVÉN, B. (1949) Notes on the fractionation and colorimetric assay of commercial heparin. *Acta chem scand*, **3**, 589

SNELLMAN, O., SYLVÉN, B. and JULÉN, C. (1951) Analysis of the native heparin-lipoprotein complex including the identification of a heparin complement (heparin co-factor) obtained from extracts of tissue mast cells. *Biochim. biophys. Acta*, **7**, 98.

SOHL, A. T., GOLDBLATT, H. and BROWN, H B. (1930) The pathological effects upon rats of excess irradiated ergosterol *J. clin. Invest*, **8**, 505.

SORENSEN, M and HAUGAARD, G. (1933) Die Anwendbarkeit der Orcinreaktion zur Bestimmung der Art und Menge von Kohlenhydraten in Eiweisstoffen. *Biochem Z*, **260**, 247.

SPEAKMAN, J B and HIRST, M. C (1931) Constitution of the keratin molecule. *Nature (Lond)*, **128**, 1073.

SPEAKMAN, J B. and HIRST, M. C. (1932) Constitution of the keratin molecule. *Nature (Lond.)*, **129**, 938.

SPRINGALL, H D (1954) *The Structural Chemistry of Proteins* London, Butterworth.

STEARNS, M. L. (1940a) Studies on development of connective tissue in transparent chambers in rabbit's ear. *Amer. J. Anat.*, **66**, 133.

STEARNS, M. L. (1940b) Studies on development of connective tissue in transparent chambers in rabbit's ear *Amer. J. Anat.*, **67**, 55.

STEIN, W. H and MILLER, E C. (1938) The composition of elastin *J biol. Chem.*, **125**, 599.

STIASNY, E. and BALÁNYI, D (1927) Über das Verhalten basischer Chromchloridbruhen. *Collegium*, p 86.

STRANDBERG, L. (1950) The preparation of chondroitin sulphuric acid. *Acta physiol scand*, **21**, 222.

STRONG, F. M. (1956) Lathyrism and odoratism. *Nutr Rev*, **14**, 65.

STUCKE, K. (1950) Über das elastische Verhalten der Achillessehne im Belastungsversuch. *Arch. klin. Ch*[illegible] 579.

SWERDLOW, M. and STROMBERG, [illegible] llagen p[illegible] by electron [illegible] J. [illegible]

SYLVÉN, B. (1938) Über das Vorkommen von metachromatischer Substanz in wachsendem Gewebe und ihre Bedutung *Klin. W'schr*, **17**, 1545.
SYLVÉN, B (1941) Über das Vorkommen von hochmolekularen Esterschwefelsäuren im Granulationsgewebe und bei der Epithelregeneration. *Acta chir. scand*, **86**, Suppl. 66
SYLVÉN, B (1950) The cytoplasm of living tissue mast cells in visual phase-contrast. *Exp Cell Res.*, **1**, 492.
SYLVÉN, B. (1951) On the cytoplasmic constituents of normal tissue mast cells *Exp. Cell Res*, **2**, 252
SYLVÉN, B (1954) Metachromatic dye-substrate interactions. *Quart. J micr Sci*, **95**, 327.
SYLVÉN, B (1954) On polysaccharides in healing wounds and tumours *Acta Un. int. Cancr*, **10**, 169
SYLVÉN, B (1956) The ground substance of connective tissue and cartilage. In *Biochemistry and Physiology of Bone*, p. 53 Edited by G Bourne. New York, Academic Press
SULVÉN, B. and MALMGREN, H (1952) On the alleged metachromasia of hyaluronic acid. *Lab. Invest*, **1**, 413.
TAKAHASHI, T. and YOKOYAMA, W (1954) Physicochemical studies on the skin and leather of marine animals XII The content of hydroxyproline in the collagen of different fish skins *Bull. Jap Soc sci Fish.*, **20**, 525.
TOMLIN, S G (1955) On the structure of collagen fibrils *Proceedings of the International Wool Textile Research Conference, Australia* (In the Press)
TOMLIN, S G and WORTHINGTON, C R (1956) Low angle X-ray diffraction patterns of collagen *Proc roy Soc*, *Ser A*, **235**, 189
TRISTRAM, G R. (1953) *The Proteins*, vol IA, p 221 Edited by H Neurath and K. Bailey New York, Academic Press
TSVETKOV, V. N (1951) Study of diffusion in liquids, using a polarizing interferometer. *J. exp. theor. Phys*, **21**, 701
TUSTANOWSKI, A. A., ZAIDES, A L, ORLOVSKAYA, G V and MIKHAILOR, A. N. (1954) New results on the structure of collagen *C R Acad Sci. U.R.S S.*, **97**, 121.
UNDENFRIEND, S and COOPER, J. R (1952) The chemical estimation of tyrosine and tyramine *J biol Chem*, **196**, 227
UNNA, P. G (1896) *Histopathology of the Diseases of the Skin* Transl N Walker New York, MacMillan
VALKO, E. (1937) *Kolloidchemische Grundlagen der Textilveredlung*, p 402 Berlin, Julius Springer.
VANAMEE, P. and PORTER, K. R Observations with the electron microscope on the solvation and reconstitution of collagen *J. exp Med*, **94**, 255

VERZÁR, F (1955) Compensat [illegible] the lifespan of rats. *3rd* [illegible] *Association, London, 1954.*

VERZÁR, F. (1955) Veränderung der thermoelastischen Eigenschafter von Sehnenfasern beim Altern *Experientia*, **11**, 230

VERZÁR, F. (1955) Veränderungen der thermoelastischen Kontraktion von Haut und Nerv bei alternden Tieren. *Experientia*, **11**, 230

VERZÁR, F (1955) Veränderungen der thermoelastischen Kontraktion von Sehnenfasern im Alter *Helv. physiol. acta*, **13**, C 64

VERZÁR, F. (1956) Das Altern des Kollagens. Hevl physiol. acta, **14**, *207.*

WALKER, D. G. and WIRTSCHAFTER, Z. T. (1956) Histopathogenesis of aortic aneurysms in the lathyrus-fed rat. *Arch. Path. (Chicago)*, **61**, 125

WASSERMAN, F. (1956) The intercellular components of connective tissue. origin, structure and interrelationship of fibers and ground substance *Ergebn Anat. EntwGesch.*, **35**, 240

WASSERMAN, K and MAYERSON, H. S. (1951) Exchange of albumin between plasma and lymph *Amer. J. Physiol*, **165**, 15.

WATSON, M. R. and SMITH, R. H (1956) The chemical composition of earthworm cuticle *Biochem. J.*, **64**, 10P.

WEGELIUS, O. (1956) The content of mast cells in the pleural membranes, pericardium and liver capsule of cattle and horses. A comparison with earlier observations and assays of heparin and of histamine in these tissues. *Acta physiol. scand*, **35**, 365.

WEGELIUS, O. and HJELMMAN, G. (1955) Vital staining of mast cells and fibrocytes. *Acta path microbiol. scand*, **36**, 304.

WEIMER, H. E., MEHL, J. W. and WINZLER, R. J. (1950) Studies on the mucoproteins of human plasma V. Isolation and characterization of a homogeneous mucoprotein. *J. biol. Chem.*, **185**, 561.

WEIR, C. E. (1949) Rate of shrinkage of tendon collagen Heat, entropy and free energy of activation of the untreated tendon *J. Amer. Leath Chem. Ass*, **42**, 17

WEIR, C E. and CARTER, J. (1950) Rate of shrinkage of tendon collagen. *J Res. nat. Bur Stand.*, **44**, 599.

WEISS, J (1954) The nature of the reaction between orcein and elastin *J Histochem Cytochem*, **2**, 21.

WEISSMANN, B and MEYER, K. (1954) The structure of hyalobiuronic acid and of hyaluronic acid from umbilical cord *J. Amer. chem. Soc.*, **76**, 1753

WELLS, A G. (1933) The chemistry of arteriosclerosis In *Arteriosclerosis — A Survey of the Problem, p. 323* Edited by E. V. Cowdry New York, Macmillan

WILANDER, O. (1938) Studien uber Heparin. *Skand. Arch. Physiol*, **81**, 1.

WILLIAMS, G. (1956) Experimental studies in arterial ligation. *J. Path. Bact.*, **72**, 569

WINDRUM, G. M., KENT, P. W. and EASTOE, J. E. (1955) The constitution of human renal reticulin *Brit. J. exp. Path.*, **36**, 49

WISLOCKI, G. B. and FAWCETT, D. W. (1951) Some histochemical properties of mast cells and tissue eosinophils in stained spreads of normal rat mesentery *J. nat. Cancer Inst.*, **12**, 258

WOERDEMANN, H. W. (1921) Histologisch onderzook naarden fibrillaren bow van eenige cellen and weefsels *Dissertation, Amsterdam*

WOHLISCH, E. (1931) Ein optisches Lineardilatometer zur Erforschung der thermoelastischen Eigenschaften des Muskels *Z. Biol.*, **91**, 137.

WOLPERS, C. (1943) Kollagenstreifung und Grundsubstanz *Klin. Wschr.*, **22**, 624

WOLPERS, C. (1944a) Die Querstreifung der kollagenen Bindegewebsfibrille *Virchows Arch.*, **312**, 292

WOLPERS, C. (1944b) Zur electronenmikroskopische Dastellung elastischer Gewebselemente *Klin. Wschr.*, **23**, 169

WOOD, G. C. (1953) The stabilization of elastin by a polysaccharide. *Biochem. J.*, **55**, xxxivP

WOOD, G. C. (1954) Some tensile properties of elastic tissue. *Biochim. biophys. Acta*, **15**, 311

WOOD, G. C. (1955) Some tensile properties of elastic tissue. *3rd Congress of the International Gerontological Association, London, 1954.* London, Livingstone

WYCKOFF, R. W. G. (1949) The fibrous proteins of skin *Brit. J. Radiol.*, **22**, 357

ZAHN, H. (1956) Discussion to lecture of W. Grassmann, Weinheim

ZOLLINGER, H. U. (1950) Gewebsmastzellen und Heparin. *Experientia*, **6**, 384

INDEX